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EUROPEAN PATENT APPLICATION

⑰ Application number: 83300442.7

⑤① Int. Cl.³: C 12 N 15/00

⑳ Date of filing: 28.01.83

③① Priority: 01.02.82 US 344710

④③ Date of publication of application:
10.08.83 Bulletin 83/32

⑥④ Designated Contracting States:
BE CH DE FR GB IT LI LU NL SE

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⑤④ Cloning vectors.

⑤⑦ The present invention discloses multi-functional recombinant DNA cloning vectors comprising

- a) two or more functionally different origins of replication that are independently selected from the group consisting of any origin of replication that is functional in *Streptomyces*, any origin of replication that is functional in *Bacillus*, and any origin of replication that is functional in *E. coli*, and
- b) one or more DNA segments that confer resistance to at least one antibiotic when transformed into a sensitive host cell in which an origin of replication comprising said vector is functional, said host cells being susceptible to transformation, cell division, and culture, subject to the limitation that when said vector is limited to two functionally different origins of replication, neither of the origins of replication is functional in *E. coli*.

CLONING VECTORS

5 The present invention relates to novel multifunctional recombinant DNA cloning vectors comprising a functional Streptomyces origin of replication, a functional Bacillus origin of replication, a functional E. coli origin of replication, and one or more DNA segments that confer resistance to antibiotics. The invention further relates to transformants of the
10 aforementioned vectors.

15 Heretofore, the development and exploitation of recombinant DNA technology in Streptomyces, Bacillus, and E. coli has been retarded and made especially difficult in part because of the general lack of selectable multifunctional cloning vectors. The vectors to which the present invention relates are functional and selectable in Streptomyces, Bacillus, and E. coli and therefore represent a significant advance in the technical art.

20 The vectors to which the present invention relates are particularly useful because they are small, versatile, and can be transformed and selected in Streptomyces, Bacillus, or E. coli cells that are sensitive to an antibiotic for which resistance is conferred. Since over half of the clinically important
25 antibiotics are produced by Streptomyces strains, it is desirable to develop cloning systems and vectors that are applicable to that industrially important group. Moreover, since the vectors are also functional in Bacillus and E. coli, the intergeneric transfer and
30 expression of genetic material is facilitated. In this context, the vectors provide for flexibility in the

selection of host cells for the production of products by recombinant DNA techniques. This is critically important because genetic expression of vector borne genes is limited and influenced by the cellular genetic background into which the genes are transformed. These
5 host cell differences are exploited by providing vectors that can be used intergenerically. Thus, the vectors can be used to clone genes for increasing the yields of known antibiotics as well as for producing new antibiotics and antibiotic derivatives in a variety of host
10 cells including especially either or both of Streptomyces and Bacillus.

The multifunctional recombinant DNA cloning vectors to which this invention relates can not only be cloned into Streptomyces, Bacillus, and E. coli host
15 cells, but also allow for the convenient selection of transformants. Since transformation is a very low frequency event, such a functional test is a practical necessity for determining which cell(s), of among the millions of cells, has acquired the plasmid DNA. This
20 is important because DNA sequences that are non-selectable can be inserted onto the vectors and, upon transformation into a host, cells containing the vector and the particular DNA sequence of interest can be isolated by appropriate antibiotic selection.

25 For purposes of the present invention as disclosed and claimed herein, the following terms are as defined below.

Recombinant DNA Cloning Vector - any autonomously replicating agent, including but not limited
30 to plasmids, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

Transformation - the introduction of DNA into a recipient host cell that changes the genotype and consequently results in a stable and heritable change in the recipient cell.

5 Transformant - a recipient host cell that has undergone transformation.

Multifunctional - denotes intergeneric functionality.

10 Sensitive Host Cell - a host cell that cannot grow in the presence of a given antibiotic without a DNA segment that confers resistance thereto.

Restriction Fragment - any linear portion or whole of a plasmid generated by the action of one or more restriction enzymes on the plasmid.

15 Insertional Isomer - one of the two or more possible recombinant DNA molecules formed when a DNA fragment is inserted at one of two or more compatible sites on the recipient DNA.

20 Plasmid pLR2 1.6 kb BamHI Restriction Fragment - the same 1.6 kb BamHI thiostrepton resistance conferring fragment contained in plasmid pIJ6.

Plasmid pLR1 3.4 kb BamHI Restriction Fragment - the same 3.4 kb BamHI neomycin resistance conferring fragment contained in plasmid pIJ2.

25 Amp^R - the ampicillin resistant phenotype.
 Tet^S - the tetracycline sensitive phenotype.
 Thio^R - the thiostrepton resistant phenotype.
 Neo^R - the neomycin resistant phenotype.
 Cm^R - the chloramphenicol resistant phenotype.
 Kn^R - the kanamycin resistant phenotype.

The present invention specifically relates to multifunctional recombinant DNA cloning vectors comprising:

- 5 a) two or more functionally different origins of replication that are independently selected from the group consisting of any origin of replication that is functional in Streptomyces, any origin of replication that is functional in Bacillus, and any origin of replication that is functional in E. coli,
10 and
- b) one or more DNA segments that confer resistance to at least one antibiotic when transformed into a sensitive host cell in which an origin of replication comprising said vector
15 is functional, said host cells being susceptible to transformation, cell division, and culture,
- subject to the limitation that when said vector is limited to two functionally different origins of
20 replication, neither of the origins of replication are functional in E coli.

The invention further relates to transformants of the aforementioned vectors.

- 25 The vectors are constructed by ligating at least two of 1) a restriction fragment comprising both an origin of replication that is functional in Streptomyces and also one or more DNA segments that confer resistance to at least one antibiotic when transformed

into Streptomyces, 2) a restriction fragment comprising both an origin of replication that is functional in Bacillus and also one or more DNA segments that confer resistance to at least one antibiotic when transformed into Bacillus, 3) a replicon containing and antibiotic resistance conferring restriction fragment of an E. coli plasmid. Ligation of 1) and 2) results in a plasmid that is functional in both Streptomyces and Bacillus. Ligation of 3) to the plasmid resulting from ligation of 1) and 2) yields a plasmid that is functional in each of Streptomyces, Bacillus, and E. coli.

A preferred first fragment, exemplified herein for illustrative purposes by the ~4.4 kb BamHI restriction fragment of plasmid pEL105, is constructed by ligating the thiostrepton resistance conferring ~1.6 kb BamHI restriction fragment of plasmid pLR2 onto the ~2.8 kb BamHI restriction fragment of plasmid pEL103. The latter fragment contains an origin of replication that is functional in Streptomyces and thus is especially useful for constructing the present invention. A preferred second fragment is the ~4.6 kb BamHI restriction fragment of plasmid pHI-16. The plasmid pHI-16 fragment contains a Bacillus functional origin of replication and also DNA segments that confer antibiotic resistance in Bacillus. Ligation of the

first and second restriction fragments results in the novel illustrative multifunctional plasmid pBS2.

Ligation of BamHI restricted plasmid pBS2 with BamHI restricted plasmid pBR322 results in the novel multifunctional illustrative plasmid pBS49. Plasmids of the latter type are highly versatile and can be used in each of Streptomyces, Bacillus, and E. coli.

Plasmid pEL103, which contains an origin of replication that is functional in Streptomyces, is approximately 20.0 kb and contains several restriction sites which are particularly advantageous for molecular cloning. Since the origin of replication of plasmid pEL103 has been localized to within the ~2.8 kb BamHI restriction fragment, a variety of different origin of replication containing fragments can be generated by digesting the plasmid with restriction enzymes that cut outside the ~2.8 kb BamHI region. A detailed restriction site and functional map of plasmid pEL103 is presented in Figure 1 of the accompanying drawings. For purposes of the present application, Figure 1 and all subsequent figures are not drawn to scale.

Plasmid pEL103 can be conventionally isolated from Streptomyces granulosus No. A39912.13/pEL103, a strain deposited and made part of the permanent stock culture collection of the Northern Regional Research Laboratory, Peoria, Illinois. It is available to the public, as a preferred source and stock reservoir of the plasmid, under the accession number NRRL 12549.

Although many different origin of replication containing fragments of plasmid pEL103 can be constructed, the ~2.8 kb BamHI restriction fragment is

most preferred for purposes of constructing the present invention. The ~2.8 kb fragment is ligated to one or more antibiotic resistance conferring DNA fragments which are exemplified herein, for illustrative purposes, by the thiostrepton resistance conferring ~1.6 kb BamHI restriction fragment of plasmid pLR2 and the neomycin resistance conferring ~3.4 kb BamHI restriction fragment of plasmid pLR1.

Plasmid pLR2, the source of the thiostrepton resistance conferring fragment, is approximately 18.7 kb and is constructed by ligating HindIII treated plasmid pIJ6, disclosed in Thompson et al., 1980, Nature 286:525, to HindIII treated plasmid pBR322. Plasmid pLR1, the source of the neomycin resistance conferring fragment, is approximately 14.8 kb and is similarly constructed, except that plasmid pIJ2, disclosed in Thompson et al., 1980, is used instead of plasmid pIJ6. Both plasmids pLR2 and pLR1 are functional in E. coli and therefore can be amplified and isolated conveniently for subsequent manipulation. A restriction site and functional map of each of plasmids pLR1 and pLR2 is presented respectively in Figures 2-3 of the accompanying drawings.

The thiostrepton resistance conferring ~1.6 kb BamHI fragment and the neomycin resistance conferring ~3.4 kb BamHI fragment are ligated to the ~2.8 kb origin of replication containing BamHI fragment of plasmid pEL103 to produce plasmids useful as starting materials for constructing the present invention. The plasmid starting materials are of two orientations depending upon the orientation of the particular resistance

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conferring DNA fragment. Thus, ligation of the ~1.6 kb BamHI fragment of plasmid pLR2 onto the ~2.8 kb BamHI fragment of plasmid pEL103 results in useful starting material plasmids pEL107 and pEL105; ligation of the
5 ~3.4 kb BamHI fragment of plasmid pLR1 results in useful starting material plasmids pEL109 and pEL110; and ligation of both of the fragments results in useful starting material plasmids pEL113, pEL114, pEL115, and pEL116. The aforementioned plasmid starting materials can be partially BamHI digested to generate restriction
10 fragments that are useful for constructing the present vectors as described herein below.

Plasmid pHI-16 contains both an origin of replication that is functional in Bacillus and also DNA segments that respectively confer resistance to anti-
15 biotics chloramphenicol and kanamycin. Therefore, plasmid pHI-16 is an excellent starting material for purposes of the present invention. Plasmid pHI-16 is ~4.6 kb and results from an in vivo deletion of known chimeric plasmid pBD12 (disclosed in Gryczan et al.,
20 1980, J. Bacteriology 141 (1):246) and can be conventionally isolated from Bacillus subtilis M1112/ pHI-16, a constructed strain deposited and made part of the permanent stock culture collection of the Northern Regional Research Laboratory, Peoria, Illinois. It is
25 available to the public, as a preferred source and stock reservoir of the plasmid, under the accession number NRRL B-12597. A detailed restriction site and functional map of plasmid pHI-16 is presented in Figure 4 of the accompanying drawings. For simplicity,
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only three MboII and TaqI restriction sites are shown for plasmid pHI-16 and the pHI-16 fragments illustrated herein.

5 Various derivatives of plasmid pHI-16 can also be used as starting materials. For example, deletion of the ~.7 kb HpaII restriction fragment of plasmid pHI-16 results in the novel plasmid pHI-18. Plasmid pHI-18 is ~3.9 kb and contains a chloramphenicol resistance conferring DNA segment as well as an origin of replication that is functional in Bacillus.
10 A restriction site and functional map of plasmid pHI-18 is presented in Figure 5 of the accompanying drawings. For simplicity, only three MboII and TaqI restriction sites are shown for plasmid pHI-18 and the pHI-18 fragments illustrated herein.

15 The BamHI digestion of plasmids pHI-16 and pHI-18 results respectively in ~4.6 kb and ~3.9 kb fragments which, with respect to Bacillus, each contain an origin of replication and also DNA conferring antibiotic resistance. Therefore, multifunctional cloning
20 vectors, illustrative of the present invention, can be readily constructed by ligating the ~3.9 kb BamHI restriction fragment of plasmid pHI-18 or the ~4.6 kb BamHI restriction fragment of plasmid pHI-16 with the ~4.4 kb BamHI restriction fragment of plasmid pEL105
25 disclosed above. The resulting multifunctional plasmids are herein respectively designated as plasmids pBS1 and pBS2. Ligation of the ~4.6 kb BamHI restriction fragment of plasmid pHI-16 with the ~6.2 kb BamHI restriction fragment of plasmid pEL110 results in the novel
30 multifunctional plasmid pBS5. Similarly, ligation of

the plasmid pHI-16 BamHI fragment with the ~7.8 kb
BamHI restriction fragment of plasmid pEL113 results in
the multifunctional plasmid pBS7. A detailed restric-
tion site and functional map of each of plasmids pBS1,
5 pBS2, pBS5, and pBS7 are presented respectively in
Figures 6, 7, 13, and 14 of the accompanying drawings.

The present vectors such as, for example,
plasmid pBS1, pBS2, pBS5 and pBS7, can be ligated to a
functional replicon containing and antibiotic resis-
tance conferring restriction fragment of an E. coli
10 plasmid such as, for example, plasmid pBR322, pBR324
(disclosed in Bolivar, F., 1978, Gene 4:121), pBR325
(disclosed in Bolivar, F., 1978), pBR328 (disclosed in
Soberon, X., 1980, Gene 9:287), or the like, to produce
novel multifunctional plasmids for use in E. coli,
15 Streptomyces, and Bacillus. These constructions,
exemplified for illustrative purposes by plasmids pBS9
and pBS10, are particularly advantageous because ampli-
fication and manipulation of plasmids can be done
faster and more conveniently in E. coli than in either
20 Streptomyces or Bacillus. Thus, after desired recombi-
nant DNA procedures are accomplished within the E. coli
host system, the particular DNA can be removed, re-
constructed to plasmid form (if necessary), and then
transformed into a Streptomyces or Bacillus host cell.

25 Although the Streptomyces functional origin
of replication that is exemplified in the present
multifunctional pBS plasmids is contained in the ~2.8 kb
BamHI fragment of plasmid pEL103, analogous pEL103
fragments can also be used provided that the origin of
30 replication is present. Such analogous plasmid pEL103

restriction fragments include, but are not limited to, the PstI, SphI, BglII, ClaI, XhoI, and the other BamHI fragments. Moreover, a particular antibiotic resistance conferring DNA segment is not limited to a single position on a plasmid pEL103 restriction fragment but can be ligated or inserted at varying sites provided that the origin of replication or other critical plasmid controlled physiological functions are not disrupted. Therefore, many different plasmid pEL103 restriction fragments and derivatives can be used in place of those exemplified herein. Those skilled in the art understand or can readily determine which sites are advantageous for the ligation or insertion of a particular DNA segment.

While the ~4.6 and 3.9 kb BamHI restriction fragments of plasmids pHI-16 and pHI-18 respectively are preferred for constructing the present multifunctional plasmids, many different origin of replication containing and antibiotic resistance conferring fragments of plasmids pHI-16 and pHI-18 can also be used. Analogous single cut restriction fragments of plasmids pHI-16 and pHI-18 include, for example, the BglII, XbaI, EcoRI, PvuII, and AvaI fragments of plasmid pHI-16 and the XbaI, EcoRI, PvuII, and AvaI fragments of plasmid pHI-18. In addition, the antibiotic resistance conferring DNA segments, contained in the plasmid pHI-16 and pHI-18 fragments, are not limited to a single position. Rather, the segments can be positioned and inserted and ligated at varying sites provided that the origin of replication or other critical plasmid con-



trolled physiological functions are not disrupted. Those skilled in the art understand or can readily determine which sites are advantageous for the ligation or insertion of a particular DNA segment. Therefore, a large number of restriction fragments can be constructed that both express antibiotic resistance and also contain an origin of replication that is functional in Bacillus. Such fragments can be used in place of those exemplified herein for constructing additional multifunctional vectors that are also within the scope of the present invention.

Antibiotic resistance conferring DNA segments can also be ligated onto other Bacillus functional plasmids such as, for example, plasmid pBD15 and plasmids (disclosed in NIH Publication No. 82-99, 1981, Recombinant DNA Technical Bulletin 4(4):143) pPL10, pPL7065, pIM13, pPL576, pBC16, pAM77, pC194, pC221, pC223, pE194, pSA2100, pSA0501, TP2, pUB110, pUB112, pBD6, pBD8, pBD9, pBD10, pBD11, pBD12, pBD20, pBD35, pBD64, pOG1196, pHV12, pHV14, pHV33, pOG2165, and pGrTI. The resulting plasmids confer antibiotic resistance, contain an origin of replication that is functional in Bacillus, and thus can be used in place of plasmids pHI-16 and pHI-18 for purposes of the present invention. Therefore, the present invention is not limited to the illustrative multifunctional vectors that require plasmids pHI-16 or pHI-18 for construction.

Although the thiostrepton, neomycin, kanamycin, and chloramphenicol antibiotic resistance conferring DNA segments are, for illustrative purposes, respectively exemplified by the ~1.6 kb BamHI restric-

tion fragment of plasmid pLR2, the ~3.4 kb BamHI restriction fragment of plasmid pLR1, the ~4.6 BamHI restriction fragment of plasmid pHI-16, and the ~3.9 BamHI restriction fragment of plasmid pHI-18, those skilled in the art can construct and substitute either individually or in combination, other DNA segments that also confer resistance to the aforementioned antibiotics. Other thiostrepton resistance conferring DNA segments of plasmid pLR2 include, for example, the ~13 kb PstI restriction fragment and also the BclI subfragment of the ~1.6 kb BamHI restriction fragment. Other neomycin resistance conferring DNA segments of plasmid pLR1 include, for example, the ~3.5 kb PstI restriction fragment and also the larger of the SstI-KpnI subfragments of the ~3.4 kb BamHI restriction fragment. Other kanamycin resistance conferring DNA segments of plasmid pHI-16 include, for example, the ~.74 kb HpaII fragment and also the larger of the XbaI-PvuII, AvaI-EcoRI, EcoRI-PvuII, and BamHI-EcoRI fragments. Other chloramphenicol resistance conferring DNA segments of plasmid pHI-18 include, for example, the ~.84 kb HpaII fragment and also the larger of the EcoRI-PvuII, BamHI-EcoRI, and XbaI-PvuII fragments.

Additional DNA segments conferring resistance to the above or to different antibiotics such as, for example, hygromycin, bacteriocin, viamycin, streptomycin, tylosin, and erythromycin, can also be used for purposes of the present invention. Moreover, various functional derivatives of the above described antibiotic resistance conferring DNA segments can be constructed by adding, eliminating, or substituting nucleotides in

accordance with the genetic code. Those skilled in the art will understand that ligation of these, or any of the other antibiotic resistance conferring DNA segments, with Streptomyces and Bacillus origins of replication containing restriction fragment, results in multifunctional vectors that are within the scope of the present invention.

The above described origin of replication containing restriction fragments and also the antibiotic resistance conferring DNA segments can be conveniently modified to facilitate subsequent ligation. For example, addition of molecular linkers provides for the construction of specific restriction sites that are useful for ligation or for other purposes known in the art. Moreover, the various restriction fragments can also be modified by adding, eliminating, or substituting nucleotides to alter characteristics and to provide a variety of unique or additional restriction sites. Those skilled in the art understand nucleotide chemistry and the genetic code and thus which nucleotides are interchangeable and which DNA modifications are desirable for a specific purpose.

The recombinant DNA cloning vectors of the present invention are not limited for use in a single species or strain of either Streptomyces or Bacillus. To the contrary, the vectors are broadly applicable and can be transformed into host cells of many Bacillus and Streptomyces taxa, particularly the restrictionless strains thereof. Moreover, with respect to Streptomyces, many of the strains are economically important

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and produce antibiotics such as aminoglycoside, macro-
lide, β -lactam, polyether, and glycopeptide antibiotics.
Restrictionless strains are readily selected and iso-
lated from Streptomyces and Bacillus taxa by conven-
5 tional procedures and extensions of principles well
known in the art (Lomovskaya et al., 1980, Microbio-
logical Reviews 44:206). Host cells of restrictionless
strains lack restriction enzymes and therefore do not
cut or degrade plasmid DNA upon transformation. For
10 purposes of the present application, host cells con-
taining restriction enzymes that do not cut any of the
restriction sites of the present vectors are also con-
sidered restrictionless.

Preferred host cells of restrictionless
15 strains of Streptomyces taxa that produce aminoglyco-
side antibiotics and in which the present vectors are
especially useful and can be transformed, include
restrictionless cells of, for example: S. kana-
myceticus (kanamycins), S. chrestomyceticus (amino-
sidine), S. griseoflavus (antibiotic MA 1267), S.
20 microsporeus (antibiotic SF-767), S. ribosidificus
(antibiotic SF733), S. flavopersicus (spectinomycin),
S. spectabilis (actinospectacin), S. rimosus forma
paromomycinus (paromomycins, catenulin), S. fradiae
var. italicus (aminosidine), S. bluensis var. bluensis
25 (bluensomycin), S. catenulae (catenulin), S. olivo-
reticuli var. cellulophilus (destomycin A), S. tene-
brarius (tobramycin, apramycin), S. lavendulae (neomy-
cin), S. albogriseolus (neomycins), S. albus var. meta-
mycinus (metamycin), S. hygroscopicus var. sagamiensis
30 (spectinomycin), S. bikiniensis (streptomycin), S.

griseus (streptomycin), S. erythrochromogenes var. narutoensis (streptomycin), S. poolensis (streptomycin), S. galbus (streptomycin), S. rameus (streptomycin), S. olivaceus (streptomycin), S. mashuensis (streptomycin), S. hygrosopicus var. limoneus (validamycins), S. rimofaciens (destomycins), S. hygrosopicus forma glebosus (glebomycin), S. fradiae (hybrimycins neomycins), S. eurocidicus (antibiotic Al6316-C), S. aquacanus (N-methyl hygromycin B), S. crystallinus (hygromycin A), S. noboritoensis (hygromycin), S. hygrosopicus (hygromycins), S. atrofaciens (hygromycin), S. kasugaspinus (kasugamycins), S. kasugaensis (kasugamycins), S. netropsis (antibiotic LL-AM31), S. lividus (lividomycins), S. hofuensis (seldomycin complex), and S. canus (ribosyl paromamine).

Preferred host cells of restrictionless strains of Streptomyces taxa that produce macrolide antibiotics and in which the present vectors are especially useful and can be transformed, include restrictionless cells of, for example: S. caelestis (antibiotic M188), S. platensis (platenomycin), S. rochei var. volubilis (antibiotic T2636), S. venezuelae (methymycins), S. griseofuscus (bundlin), S. narbonensis (josamycin, narbomycin), S. fungicidicus (antibiotic NA-181), S. griseofaciens (antibiotic PA133A, B), S. roseocitreus (albocycline), S. brunoegriseus (albocycline), S. roseochromogenes (albocycline), S. cinerochromogenes (cineromycin B), S. albus (albomycetin), S. felleus (argomycin, picromycin), S. rochei (lankacidin, borrelidin), S. violaceoniger (lankacidin), S. griseus (borrelidin), S. maizeus

(ingramycin), S. albus var. coilmyceticus (coleimycin),
S. mycarofaciens (acetyl-leukomycin, espinomycin), S.
hygroscopicus (turimycin, relomycin, maridomycin,
tylosin, carbomycin), S. griseospiralis (relomycin), S.
5 lavendulae (aldgamycin), S. rimosus (neutramycin), S.
deltae (deltamycins), S. fungicidicus var. espino-
myceticus (espinomycins), S. furdicidicus (mydeca-
mycin), S. ambofaciens (foromacidin D), S. eurocidicus
(methymycin), S. griseolus (griseomycin), S. flavo-
10 chromogenes (amaromycin, shincomycins), S. fimbriatus
(amaromycin), S. fasciculus (amaromycin), S. erythreus
(erythromycins), S. antibioticus (oleandomycin), S.
olivochromogenes (oleandomycin), S. spinichromogenes
var. suraçaoensis (kujimycins), S. kitasatoensis
15 (leucomycin), S. narbonensis var. josamyceticus (leuco-
mycin A3, josamycin), S. albogriseolus (mikonomycin),
S. bikiniensis (chalcomycin), S. cirratus (cirramycin),
S. djakartensis (niddamycin), S. eurythermus (angola-
mycin), S. fradiae (tylosin, lactenocin, macrocin), S.
goshikiensis (bandamycin), S. griseoflavus (acumycin),
20 S. halstedii (carbomycin), S. tendae (carbomycin), S.
macrosporeus (carbomycin), S. thermotolerans (carbo-
mycin), and S. albireticuli (carbomycin).

Preferred host cells of restrictionless
strains of Streptomyces taxa that produce β -lactam
25 antibiotics and in which the present vectors are
especially useful and can be transformed, include
restrictionless cells of, for example: S. lipmanii
(A16884, MM4550, MM13902), S. clavuligerus (A16886B,
clavulanic acid), S. lactamdurans (cephamycin C), S.
30 griseus (cephamycin A, B), S. hygroscopicus (deacetoxy-

cephalosporin C), S. wadayamensis (WS-3442-D), S. chartreusis (SF 1623), S. heteromorphus and S. panayensis (C2081X); S. cinnamomensis, S. fimbriatus, S. halstedii, S. rochei and S. viridochromogenes (cephamycins A, B); S. cattleya (thienamycin); and S. olivaceus, S. flavovirens, S. flavus, S. fulvoviridis, S. argenteolus, and S. sioyaensis (MM 4550 and MM 13902).

Preferred host cells of restrictionless strains of Streptomyces taxa that produce polyether antibiotics and in which the present vectors are especially useful and can be transformed, include restrictionless cells of, for example: S. albus (A204, A28695A and B, salinomycin), S. hygroscopicus (A218, emeracid, DE3936), A120A, A28695A and B, etheromycin, dianemycin), S. griseus (grisorixin), S. conglobatus (ionomycin), S. eurocidicus var. asterocidicus (laidlomycin), S. lasaliensis (lasalocid), S. ribosidificus (lonomycin), S. cacaoi var. asoensis (lysocellin), S. cinnamomensis (monensin), S. aureofaciens (narasin), S. callinarius (RP 30504), S. longwoodensis (lysocellin), S. flaveolus (CP38936), S. mutabilis (S-11743a), and S. violaceoniger (nigericin).

Preferred host cells of restrictionless strains of Streptomyces taxa that produce glycopeptide antibiotics and in which the present vectors are especially useful and can be transformed, include restrictionless cells of, for example: S. orientalis and S. haranomachiensis (vancomycin); S. candidus (A-35512, avoparcin), and S. eburosporeus (LL-AM 374).

Preferred host cells of other Streptomyces restrictionless strains in which the present vectors

are especially useful and can be transformed, include restrictionless cells of, for example: S. coelicolor, S. granuloruber, S. roseosporus, S. lividans, S. espinosus, and S. azureus.

5 Preferred host cells of restrictionless strains of Bacillus in which the present vectors are especially useful and can be transformed, include restrictionless cells of, for example, B. subtilis, B. subtilis Mill2, B. thuringiensis, B. thuringiensis var. israeliensis, B. cereus, B. anthracis, B. piliformis,
10 B. tropicus, B. alvei, B. megaterium, B. pumilus, B. licheniformis, B. polymyxa, B. macerans, B. circulans, B. stearothermophilus, B. coagulans, B. firmus, B. brevis, B. sphaericus, B. pasteurii, B. fastidiosus,
15 B. larvae, B. lentimorbus, B. apiarius, B. amylo-liquifaciens, B. laterosporus, and B. popillae.

In addition to the representative Streptomyces and Bacillus host cells described above, the present vectors are also useful and can be transformed into cells of restrictionless strains of other taxa
20 such as, for example: Staphylococcus, Staphylococcus aureus, Streptococcus, related Actinomycetes, including Streptosporangium, Actinoplanes, Nocardia, and Micromonospora, and Lactobacillus. Furthermore, the multi-
25 functional vectors that contain an E. coli replicon can also be transformed into E. coli. Thus, the vectors of the present invention have wide application and are useful and can be transformed into host cells of a variety of organisms.

30 While all the embodiments of the present invention are useful, some of the present recombinant

DNA cloning vectors and transformants are more preferred than others. Accordingly, preferred vectors are plasmids pBS1, pBS2, pBS5, pBS7 and pBS9; and preferred transformants are Streptomyces ambofaciens/pBS1, S.
5 ambofaciens/pBS2, S. ambofaciens/pBS5, S. ambofaciens/-pBS7, S. ambofaciens/pBS9, Bacillus subtilis MI112/pBS1, B. subtilis/MI112/pBS2, B. subtilis/MI112/pBS5, B. subtilis/MI112/pBS7, B. subtilis/MI112/pBS9, and E. coli K12 HB101/pBS9.

10 The recombinant DNA cloning vectors and transformants of the present invention have broad utility and help fill the need for suitable multi-functional cloning vehicles for use in Streptomyces, Bacillus, E. coli, and related organisms. Moreover,
15 the ability of the present vectors to confer resistance to antibiotics that are toxic to non-transformed host cells, also provides a functional means for selecting transformants. This is important because of the practical necessity for determining and selecting the
20 particular cells that have acquired vector DNA. Additional DNA segments, that lack functional tests for their presence, can also be inserted onto the present vectors and then transformants containing the non-selectable DNA can be isolated by appropriate anti-
25 biotic selection. Such non-selectable DNA segments can be inserted at any site, except within regions necessary for plasmid function and replication, and include, but are not limited to, genes that specify antibiotic modification enzymes and regulatory genes of all types.

30 More particularly, a non-selectable DNA segment that comprises a gene can be inserted on a plasmid

such as for example, illustrative plasmid pBS9, at the central SalI restriction site of the ~ 1.6 kb BamHI resistance conferring fragment. Such an insertion inactivates the thiostrepton resistance gene and thus allows for the easy identification of Streptomyces transformants containing the recombinant plasmid. This is done by first selecting for neomycin resistance and, secondarily, identifying those neomycin resistant Streptomyces transformants that are not resistant to thiostrepton. In a similar manner, insertion of a DNA segment of interest at, for example, the internal BamHI restriction site of the ~ 3.4 kb BamHI resistance conferring fragment inactivates the neomycin resistance gene. Thus, Streptomyces transformants carrying this recombinant plasmid also are identified easily by first selecting for thiostrepton resistance and, secondarily, identifying those thiostrepton resistant transformants that are not resistant to neomycin. Therefore, the ability to select for antibiotic resistance in Streptomyces allows for the efficient isolation of the extremely rare cells that contain the particular non-selectable DNA of interest.

In addition, a non-selectable DNA segment that comprises a gene can also be inserted on a plasmid such as for example, illustrative plasmid pBS9, at the BglII restriction site of the kanamycin resistance conferring fragment. Such an insertion inactivates the kanamycin resistance gene and thus allows for the easy identification of Bacillus transformants containing the recombinant plasmid. This is done by first selecting for chloramphenicol resistance and, secondarily, iden-

tifying those chloramphenicol resistant Bacillus transformants that are not resistant to kanamycin. In a similar manner, insertion of a DNA segment of interest at, for example, the internal MboII restriction site of the chloramphenicol resistance conferring fragment
5 inactivates the chloramphenicol resistance gene. Thus, Bacillus transformants carrying this recombinant plasmid also are identified easily by first selecting for kanamycin resistance and, secondarily, identifying those kanamycin resistant transformants that are not
10 resistant to chloramphenicol. Therefore, the ability to select for antibiotic resistance in Bacillus also allows for the efficient isolation of the extremely rare cells that contain the particular non-selectable DNA of interest. Similar selections can also be done
15 in E. coli.

The functional test for antibiotic resistance, as described herein above, is also used to locate DNA segments that enhance copy number or act as control elements and direct expression of an individual
20 antibiotic resistance gene. Such segments, including but not limited to, promoters, attenuators, repressors, inducers, ribosomal binding sites, and the like, are used to control the expression of other genes in cells of Streptomyces, Bacillus, E. coli, and related organisms.

25 The thiostrepton, neomycin, kanamycin, and chloramphenicol resistance conferring vectors of the present invention are also useful for insuring that linked DNA segments are stably maintained in host cells over many generations. These genes or DNA fragments,

30

covalently linked to the thiostrepton, neomycin, kanamycin, or chloramphenicol resistance conferring fragment and propagated either in Streptomyces, Bacillus, E. coli, or in the cells of related organisms, are
5 maintained by exposing the transformants to levels of thiostrepton, neomycin, kanamycin, or chloramphenicol that are toxic to non-transformed cells. Therefore, transformants that lose the vector, and consequently any covalently linked DNA, cannot grow and are eliminated from the culture. Thus, the vectors of the
10 present invention can stabilize and maintain any DNA sequence of interest, particularly in Streptomyces, Bacillus, and E. coli.

The cloning vectors and transformants of the
15 present invention provide for the cloning of genes to improve yields of various products that are currently produced in Streptomyces and related cells. Examples of such products include, but are not limited to, Streptomycin, Tylosin, Cephalosporins, Actaplanin,
20 Narasin, Monensin, Apramycin, Tobramycin, Erythromycin, and the like. The present invention also provides selectable vectors that are useful for cloning, characterizing, and reconstructing DNA sequences that code for commercially important proteins such as, for example,
25 human insulin, human proinsulin, glucagon, interferon, human growth hormone, bovine growth hormone, and the like; for enzymatic functions in metabolic pathways leading to commercially important processes and compounds; or for control elements that improve gene
30 expression. These desired DNA sequences include, but

are not limited to, DNA that codes for enzymes that catalyze synthesis of derivatized antibiotics such as, for example, Streptomycin, Cephalosporin, Tylosin, Actaplanin, Narasin, Monensin, Apramycin, Tobramycin, and Erythromycin derivatives, or for enzymes that mediate and increase bioproduction of antibiotics or other products. The capability for inserting and stabilizing such DNA segments thus allows for increasing the yield and availability of antibiotics.

The present multifunctional cloning vectors also allow for the genetic expression in Streptomyces of products now bioproduced in E. coli and Bacillus. This is especially advantageous because large scale Streptomyces fermentation is better known and understood than is fermentation of either Bacillus or E. coli. In fact, large scale commercial fermentation of E. coli is still highly experimental and sometimes difficult. The present invention circumvents this problem by providing the alternative of producing compounds now biosynthesized in E. coli such as, for example, human insulin, human proinsulin, glucagon, interferon, human growth hormone, bovine growth hormone, and the like, in Streptomyces. This can be done because the present vectors are highly versatile and can accommodate either or both of DNA sequences which express the aforementioned products or sequences comprising entire product producing E. coli plasmids. Thus, the present invention allows for flexibility in the choice of hosts and provides a means for the bioproduction of polypeptides and other products in Strepto-

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-25-

myces, Bacillus, and E. coli. Those skilled in the art know or can readily determine which host is most advantageous for both producing a particular product and for a given fermentation.

5 Streptomyces granuloruber No. A39912.13/-
pEL103 and Bacillus subtilis/M1112/pHI-16, as respec-
tive sources of plasmids pEL103 and pHI-16, can be
cultured in a number of ways using any of several
different media. Carbohydrate sources which are pre-
ferred in a culture medium include, for example, molas-
ses, glucose, dextrin, and glycerol, and nitrogen
sources include, for example, soy flour, amino acid
mixtures, and peptones. Nutrient inorganic salts are
also incorporated and include the customary salts
15 capable of yielding sodium, potassium, ammonia, calcium,
phosphate, chloride, sulfate, and like ions. As is
necessary for the growth and development of other
microorganisms, essential trace elements are also
added. Such trace elements are commonly supplied as
20 impurities incidental to the addition of other constitu-
ents of the medium.

Streptomyces granuloruber No. A39912.13/
pEL103 is grown under aerobic culture conditions over a
relatively wide pH range of about 5 to 9 at temper-
atures ranging from about 15° to 40°C. For production
25 of plasmid pEL103 at highest copy number, however, it
is desirable to start with a culture medium at a pH of
about 7.2 and maintain a culture temperature of about
30°C. Culturing Streptomyces granuloruber No. A39912.13/
30 pEL103, under the aforementioned conditions, results in

a reservoir of cells from which plasmid pEL103 is isolated conveniently by techniques well known in the art.

Bacillus subtilis Mill2/pHI-16 is grown under aerobic culture conditions over a relatively wide pH range of about 5.0 to 8.5 at temperatures ranging from about 25° to 45°C. For production of plasmid pHI-16 at highest copy number, however, it is desirable to start with a culture medium at a pH of about 7 and maintain a culture temperature of about 37°C. Culturing Bacillus subtilis Mill2/pHI-16, under the aforementioned conditions, results in a reservoir of cells from which plasmid pHI-16 is isolated conveniently by techniques well known in the art.

The following examples further illustrate and detail the invention disclosed herein. Both an explanation of and the actual procedures for constructing the invention are described where appropriate.

Example 1

Isolation of Plasmid pEL103

A. Culture of Streptomyces granulosus No. A39912.13/pEL103

A vegetative inoculum of Streptomyces granulosus No. A39912.13/pEL103 (NRRL 12549) was conventionally prepared by growing the strain under submerged aerobic conditions in 50 ml. of sterilized trypticase soy broth* at 35 g./l. in deionized water.

The trypticase soy broth inoculum was incubated for 48 hours at a temperature of 30°C. After

incubation, about 10 ml. of the inoculum was transferred to 500 ml. of the sterilized broth and was incubated for about 20 hours at 30°C. The pH was not adjusted. After incubation, the Streptomyces granu-
5 loruber No. A39912.13/pEL103 cells were ready for harvest and subsequent isolation of plasmid DNA.

* Trypticase soy broth is obtained from BBL Division, Becton-Dickinson & Company, Cockeysville, Maryland 21030

10 B. Plasmid Isolation

About 12 g. (wet wgt) of Streptomyces granu-
loruber No. A39912.13/pEL103 cells were centrifuged
(10 minutes, 4°C., 10,000 rpm), washed in 10% glycerol,
15 and then harvested by recentrifugation under the aforementioned conditions. About 50 ml. of TES buffer (.01M Tris(hydroxymethyl)aminoethane [Tris], .001M EDTA, 34% sucrose, pH 8) were added to the cells followed by
about 0.25 g. of lysozyme in 10 ml. of 0.25M EDTA.
20 After the mixture was incubated at 37°C. for about 15 minutes, about 0.5 ml. of 10% Triton X-100 in TE buffer (.01M Tris, .001M EDTA, pH 8) was added. The resultant mixture was then incubated at 65°C. for about 15 minutes.
After the lysate was centrifuged (45 minutes, 4°C.,
25 18,000 rpm), the supernatant was extracted four times with isoamyl alcohol and once with a chloroform-isoamyl alcohol solution (24:1). Next, .1 volume of 3M sodium acetate was added to the aqueous phase followed by 3 volumes of cold (-20°C.) 95% ethanol. The
30 ethanol precipitation was rapidly performed in a dry

ice-ethanol bath and the DNA precipitate was collected by centrifugation (15 minutes, 4°C., 10,000 rpm). The precipitate was vacuum dried and then resuspended in 1.1 ml. of STE buffer (.01M Tris, .001M EDTA, .01M sodium chloride). Centrifugation (40 hours, 15°C., 35,000 rpm) using cesium chloride gradients, with ethidium bromide, was carried out to purify the plasmid DNA. Following centrifugation, the desired plasmid pEL103 DNA band was removed and the ethidium bromide extracted by conventional procedures. After precipitation of the DNA in 3 volumes of ethanol, the thus isolated plasmid pEL103 DNA was dissolved in 1 ml. of 10 fold diluted TE buffer and was then stored at -20°C.

Example 2

15

Construction of Plasmid pLR2

A. HindIII Digestion of Plasmid pIJ6

About 20 µl. (20 µg.) of plasmid pIJ6 DNA, disclosed in Thompson et al., 1980, Nature 286:525, 5 µl. BSA(Bovine Serum albumin, 1 mg./ml.), 19 µl. water, 1 µl. of HindIII (containing 3 New England Bio Labs units) restriction enzyme*, and 5 µl. reaction mix** were incubated at 37°C. for 2 hours. The reaction was terminated by the addition of about 50 µl. of 4M ammonium acetate and 200 µl. of 95% ethanol. The resultant DNA precipitate was washed twice in 70% ethanol, dried in vacuo, suspended in 20 µl. of TE buffer, and stored at -20°C.

30

* Restriction enzymes can be obtained from the following sources:

New England Bio Labs., Inc.
32 Tozer Road
Beverly, Massachusetts 01915

Boehringer-Mannheim Biochemicals
7941 Castleway Drive
Indianapolis, Indiana 46250

5

** Reaction mix for HindIII restriction enzyme was prepared with the following composition.

600mM NaCl

100mM Tris-HCl, pH7.9

10

70mM MgCl₂

10mM Dithiothreitol

B. HindIII Digestion of Plasmid pBR322

15

About 8 μ l. (4 μ g.) of plasmid pBR322 DNA*, 5 μ l. reaction mix, 5 μ l. BSA (1 mg./ml.), 31 μ l. water, and 1 μ l. of HindIII restriction enzyme were incubated at 37°C. for 2 hours. After the reaction was terminated by incubating at 60°C. for 10 minutes, about 50 μ l. of ammonium acetate and 200 μ l. of 95% ethanol were added. The resultant DNA precipitate was washed twice in 70% ethanol, dried in vacuo, and suspended in 45 μ l. of water.

20

25

* Plasmid pBR322 can be obtained from Boehringer-Mannheim Biochemicals the address of which is disclosed in Example 2A.

C. Ligation of HindIII Digested Plasmids pIJ6 and pBR322

30

About 20 μ l. of HindIII treated plasmid pIJ6 (from Example 2A), 20 μ l. of HindIII treated plasmid pBR322 (from Example 2B), 5 μ l. BSA (1 mg./ml.), 1 μ l.

of T4 DNA ligase*, and 5 μ l. ligation mix** were incubated at 16°C. for 4 hours. The reaction was terminated by the addition of about 50 μ l. 4M ammonium acetate and 200 μ l. of 95% ethanol. The resultant DNA precipitate was washed twice in 70% ethanol, dried in vacuo, and suspended in TE buffer. The suspended DNA constituted the desired plasmid pLR2.

* T4 DNA ligase can be obtained from the following source:

New England Bio Labs., Inc.
32 Tozer Rd.
Beverly, Massachusetts 01915

** Ligation mix was prepared with the following composition.

500mM Tris-HCl, pH7.8
200mM Dithiothreitol
100mM MgCl₂
10mM ATP

Example 3

Construction of E. coli K12 HB101/pLR2

About 10 ml. of frozen competent E. coli K12 HB101 cells (Bolivar et al., 1977, Gene 2:75-93) were pelleted by centrifugation and then suspended in about 10 ml. of 0.01M sodium chloride. Next, the cells were pelleted again, resuspended in about 10 ml. of 0.03M calcium chloride, incubated on ice for 20 minutes, pelleted a third time, and finally, resuspended in 1.25 ml. of 0.03M calcium chloride. The resultant cell suspension was competent for subsequent transformation.

Plasmid pLR2 in TE buffer (prepared in Example 2C) was ethanol precipitated, suspended in 150 μ l. of 30mM calcium chloride solution, and gently mixed in a test tube with about 200 μ l. of competent E. coli K12 HB101 cells. The resultant mixture was incubated on ice for about 45 minutes and then at 42°C. for about 1 minute. Next, about 3 ml. of L-broth (Bertani, 1951, J. Bacteriology 62:293) containing 50 μ g./ml. of ampicillin was added. The mixture was incubated with shaking at 37°C. for 1 hour and then plated on L-agar (Miller, 1972, Experiments in Molecular Genetics, Cold Spring Harbor Labs, Cold Spring Harbor, New York) containing ampicillin. Surviving colonies were selected and tested for the expected phenotype (Amp^R, Tet^S), and constituted the desired E. coli K12 HB101/pLR2 transformants.

Example 4

Construction of Plasmid pLR1

Plasmid pLR1 was prepared in substantial accordance with the teaching of Example 2A-C except that plasmid pIJ2, disclosed in Thompson et al., 1980, Nature 286:525, was used in place of plasmid pIJ6. The desired plasmid pLR1 was suspended in TE buffer.

25

Example 5

Construction of E. coli K12 HB101/pLR1

The desired construction was carried out in substantial accordance with the teaching of Example 3 except that plasmid pLR1, rather than plasmid pLR2, was used for transformation. Surviving colonies were

30

selected and tested for the expected phenotype (Amp^R, Tet^S), and constituted the desired E. coli K12 HB101/pLR1 transformants.

Example 6

5 Construction of Plasmids pEL107 and pEL105

A. BamHI Digestion of Plasmid pLR2 and Isolation of the ~1.6 kb Thiostrepton Resistance Conferring Fragment

10 About 50 µg. of plasmid pLR2 DNA, 10 µl reaction mix*, 10 µl. BSA (1 mg./ml.), 29 µl. water, and 1 µl. (4 units/µl) of BamHI restriction enzyme were incubated at 37°C. for 2 hours. After adding an equal volume of 4M ammonium acetate and 2.5 volumes of 95%
15 ethanol, the mixture was cooled at -20°C. for about 18 hours to precipitate the DNA. The DNA precipitate was collected by centrifugation and then suspended in about 50 µl. of TE buffer. The desired ~1.6 kb BamHI restriction fragment was isolated conventionally from the DNA
20 suspension by gel electrophoresis. Following isolation, the fragment was resuspended in about 20 µl. of TE buffer for subsequent ligation.

25 * Reaction mix for BamHI restriction enzyme was prepared with the following composition.

1.5M NaCl
60mM Tris-HCl, p^H7.9
60mM MgCl₂

B. Partial BamHI Digestion of Plasmid pEL103

30 About 20 µg. of plasmid pEL103 DNA, 10 µl. reaction mix, 10 µl. BSA (1 mg./ml.), 39 µl. water, and

1 μ l. of BamHI restriction enzyme (prepared by diluting
2 μ l. of enzyme in 8 μ l. of water) were incubated at
ambient temperature for about 15 minutes. After adding
an equal volume of 4M ammonium acetate and 2 volumes of
95% ethanol, the mixture was cooled at -20°C . for about
5 18 hours to precipitate the DNA. The DNA precipitate
was collected by centrifugation, rinsed in 70% ethanol,
dried in vacuo, and then suspended in about 50 μ l. of
TE buffer.

10 C. Ligation

A mixture of about 20 μ g. of the partially
digested plasmid pEL103 DNA, 10 μ g. of the ~ 1.6 kb BamHI
restriction fragment of plasmid pLR2, 5 μ l. ligation
mix, 5 μ l. BSA (1 mg./ml.), 10 μ l. water, and 1 μ l. T4
15 DNA ligase were incubated at about 16°C . for about 4
hours. After adding 40 μ l. of 4M ammonium acetate and
200 μ l. of cold ethanol, the mixture was cooled to -20°C .
for about 18 hours to precipitate the DNA. The DNA
precipitate was collected by centrifugation, washed
20 with 70% ethanol, collected again, and then suspended
in 50 μ l. of medium P (Hopwood and Wright 1978, J.
Molecular and General Genetics 162:307) for subsequent
transformation.

Recombinant plasmids of various types result
25 depending upon which of the possible pEL103 restriction
fragments becomes ligated to the ~ 1.6 kb BamHI thio-
strepton resistance conferring fragment. Ligation to
the ~ 2.8 kb BamHI restriction fragment of plasmid pEL103
results in the desired ~ 4.4 kb plasmids pEL107 and
30 pEL105. Recombinant plasmids of two orientations

result because the ~ 1.6 kb BamHI resistance conferring fragment can be oriented in either direction. A restriction site and functional map of each of plasmids pEL107 and pEL105 is presented in Figure 8 of the accompanying drawings.

5 Those skilled in the art will recognize and understand that the partial BamHI digestion of plasmid pEL103 produces a mixture of different restriction fragments that can be ligated with each other and also with one or more resistance conferring DNA fragments to
10 produce several additional recombinant plasmids. Any additional plasmids that contain the ~ 2.8 kb BamHI origin of replication containing fragment can also be used to further exemplify the construction of the
15 present invention. The aforementioned additional plasmids can be conventionally transformed into appropriate host cells and then identified by restriction enzyme and gel electrophoretic analysis.

Example 7

20 Construction of *Streptomyces ambofaciens*/pEL107 and *S. ambofaciens*/pEL105

Using about 20 $\mu\text{g.}$ of the DNA from Example 6C and 1×10^9 protoplasts (prepared according to Baltz, 1978,
25 J. of General Microbiology 107:93) of *Streptomyces ambofaciens*, a strain deposited and made part of the permanent stock culture collection of the Northern Regional Research Laboratory, Peoria, Illinois, from which it is available to the public under the accession number NRRL
30 2420, the desired constructions were made in substantial accordance with the teaching of International Publica-

tion (of International Patent Application No. PCT/GB79/00095) No. WO79/01169, Example 2. The desired transformants were selected for thiostrepton resistance by overlaying the regenerating protoplasts with modified
5 R2 medium (Baltz, 1978, J. of General Microbiology 107:93) top agar containing sufficient thiostrepton to bring the final plate concentration to 50 µg./ml. The resultant Streptomyces ambofaciens/pEL107 and S. ambofaciens/pEL105 thiostrepton resistant colonies were
10 isolated according to known procedures, cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

Accordingly, vegetative inocula (10 ml.) of different isolated colonies are conventionally prepared
15 by inoculating trypticase soy broth containing sufficient thiostrepton to bring the final concentration to 50 µg./ml. Several inocula are prepared and the following procedure performed until all the desired transformant types and constitutive plasmids are isolated.
20 Thus, after cells are incubated at 30°C. until fully grown, 6 ml. of the cell containing broth are centrifuged. The resultant pellet is washed in TE buffer, pelleted again, and then suspended in 400 µl. 50mM Tris, pH 8.0. Next, about 80 µl. of .25M EDTA, 20 µl.
25 RNase, and 100 µl. (10 mg./ml. in TE) lysozyme are added. After the mixture is incubated at 37°C. for about 15 minutes, about 10 µl. of 10% Triton X-100 and 150 µl. 5M NaCl are added followed by a final incubation at 60°C. for 15 minutes. The resultant
30 lysate is centrifuged (15 minutes, 4°C., 15,000 rpm)

and then the supernatant is conventionally extracted twice with phenol, once with a chloroform-isoamyl alcohol solution (24:1), and then ethanol precipitated. The identity of the constitutive plasmids and thus the transformants is determined conventionally by restriction enzyme and gel electrophoretic analysis.

Example 8

Construction of Plasmids pEL109 and pEL110

A. Partial BamHI Digestion of Plasmid pLR1 and Isolation of the ~3.4 kb Neomycin Resistance Confer- ring Fragment

The desired partial digestion and isolation are carried out in substantial accordance with the teaching of Example 6B. The ~3.4 kb BamHI restriction fragment is suspended in about 20 μ l. of TE buffer for subsequent ligation.

B. Ligation

The ~3.4 kb BamHI neomycin resistance conferring restriction fragment is ligated to partially BamHI digested plasmid pEL103 (prepared in Example 6B) in substantial accordance with the teaching of Example 6C.

Recombinant plasmids of various types result depending upon which of the possible pEL103 restriction fragments becomes ligated to the ~3.4 kb BamHI neomycin resistance conferring fragment. Ligation to the ~2.8 kb BamHI restriction fragment of plasmid pEL103 results in the desired ~6.2 kb plasmids pEL109 and pEL110. Recom-

binant plasmids of two orientations result because the
~3.4 kb BamHI resistance conferring fragment can be
oriented in either direction. A restriction site and
functional map of each of plasmids pEL109 and pEL110
is presented in Figure 9 of the accompanying drawings.

Those skilled in the art will recognize and
understand, as described in Example 6C, that additional
recombinant plasmids containing the ~2.8 kb BamHI origin
of replication containing fragment can be generated by
the above procedure. These plasmids can be used to
further exemplify the construction of the present
invention. The aforementioned additional plasmids can
be conventionally transformed and then identified by
restriction enzyme and gel electrophoretic analysis.

Example 9

Construction of *Streptomyces ambofaciens*/pEL109 and *S. ambofaciens*/pEL110

Using about 20 µg. of the DNA from Example 8
and 1×10^8 protoplasts of *Streptomyces ambofaciens* (NRRL
No. 2420), the desired constructions are made in
substantial accordance with the teaching of International
Publication (of International Patent Application No.
PCT/GB79/00095) No. W079/01169, Example 2. The desired
transformants are selected for neomycin resistance by
overlaying the regenerating protoplasts with modified
R2 medium top agar containing sufficient neomycin* to
bring the final plate concentration to 1 µg./ml.

The resultant *Streptomyces ambofaciens*/pEL109
and *S. ambofaciens*/pEL110 neomycin resistant colonies

are isolated according to known procedures cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids. The identity of the constitutive plasmids and thus the transformants is determined in substantial accordance with the teaching of Example 7 except that neomycin (1 µg./ml.), rather than thiostrepton, is added to the trypticase soy broth.

* Antibiotic neomycin can be obtained from Sigma, St. Louis, Missouri.

Example 10

Construction of Plasmids pEL113 and pEL114

Plasmid pEL107 is isolated from Streptomyces ambofaciens/pEL107 (prepared in Example 7) according to the procedure of Example 1 and then is partially digested with BamHI restriction enzyme in substantial accordance with the teaching of Example 6B. The partial BamHI digest is then ligated, in substantial accordance with the teaching of Example 6C, with the ~3.4 kb neomycin resistance conferring BamHI fragment (prepared in Example 8A) of plasmid pLR1 to produce the desired plasmids. The insertional isomers of plasmids pEL113 and pEL114 are also produced since plasmid pEL107 has more than a single BamHI restriction site for the insertion of the neomycin resistance fragment. Recombinant plasmids of two orientations result because the ~3.4 kb BamHI neomycin resistance conferring fragment can be oriented in either direction. A restriction

site and functional map of each of plasmids pEL113 and pEL114 is presented in Figure 10 of the accompanying drawings.

Example 11

5 Construction of Streptomyces ambofaciens/pEL113 and
 S. ambofaciens/pEL114

 Using 20 µg. of the DNA from Example 10 and
10 1×10^8 protoplasts of Streptomyces ambofaciens (NRRL No.
 2420), the desired constructions are made in substantial
 accordance with the teaching of International Publication
 (of International Patent Application No. PCT/GB79/00095)
 No. W079/01169, Example 2. The desired transformants
15 are selected first for thiostrepton resistance and then
 for neomycin resistance by the methods described in
 Examples 7 and 9 above. The resultant Streptomyces
 ambofaciens/pEL113 and S. ambofaciens/pEL114 thio-
 strepton and neomycin resistant colonies are isolated
20 according to known procedures, cultured, and then
 identified by restriction enzyme and gel electrophoretic
 analysis of the constitutive plasmids. The identity of
 the constitutive plasmids and thus the transformants is
 determined in substantial accordance with the teaching
25 of Example 7 except that neomycin (1 µg./ml.) and
 thiostrepton (50 µg./ml.) are both added to the trypti-
 case soy broth.

30

Example 12Construction of Plasmids pEL115 and pEL116

5 The desired plasmids are constructed in substantial accordance with the teaching of Example 10 with the exception that plasmid pEL105, rather than plasmid pEL107, is used in the partial BamHI digestion. The insertional isomers of plasmids pEL115 and pEL116 are also produced since plasmid pEL105 has more than a single BamHI restriction site for the insertion of
10 the neomycin resistance fragment. Recombinant plasmids of two orientations result because the ~3.4 kb BamHI neomycin resistance conferring fragment can be oriented in either direction. A restriction site and functional map of each of plasmids pEL115 and pEL116 is presented
15 in Figure 11 of the accompanying drawings.

Example 13Construction of Streptomyces ambofaciens/pEL115 and S. ambofaciens/pEL116

20 Using 20 µg. of the DNA from Example 12, the desired constructions are made in substantial accordance with the teaching of Example 11. The resultant Streptomyces ambofaciens/pEL115 and S. ambofaciens/pEL116 thiostrepton and neomycin resistant colonies are
25 isolated according to known procedures, cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids in substantial accordance with the teaching of Example 11.

30

Example 14Construction of Plasmids pEL121 and pEL122

5 The desired plasmids are obtained by ligating
a partial BamHI digest of plasmid pEL107 onto BamHI
digested plasmid pBR322 in substantial accordance with
the ligation procedure of Example 6C. Plasmid pEL107
is isolated from Streptomyces ambofaciens/pEL107
(prepared in Example 7) in substantial accordance with
the teaching of Example 1 and then partially BamHI
10 digested according to the procedure of Example 6B. The
BamHI digested plasmid pBR322 is prepared in substan-
tial accordance with the procedure of Example 2B with
the exception that BamHI, rather than HindIII, restric-
tion enzyme is used. The desired plasmid DNA is col-
15 lected by centrifugation, washed with 70% ethanol,
dried in vacuo, and then suspended in 50 μ l. of TE
buffer. In addition, the insertional isomers of plasmids
pEL121 and pEL122 are also produced since plasmid
pEL107 has more than a single BamHI restriction site
20 for the insertion of the restricted plasmid pBR322.
Recombinant plasmids of two orientations result because
the restricted plasmid pBR322 can be oriented in either
direction. A restriction site and functional map of
each of plasmids pEL121 and pEL122 is presented in
25 Figure 12, of the accompanying drawings.

Example 15Construction of *E. coli* K12 HB101/pEL121 and *E. coli* K12 HB101/pEL122

5 The desired constructions are made in substantial accordance with the teaching of Example 3 with the exception that plasmids pEL121 and pEL122, rather than plasmid pLR2, are used for the transformation. Surviving colonies are first selected, tested for the expected phenotype (Amp^R, Tet^S), and then conventionally identified as the desired *E. coli* K12 HB101/pEL121 and *E. coli* K12 HB101/pEL122 transformants by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

Example 16

15

Isolation of Plasmid PHI-16A. Culture of *Bacillus subtilis* MI112/pHI-16

20 A vegetative culture of *Bacillus subtilis* MI112/pHI-16 (NRRL B-12597) was conventionally prepared by plating on PAB agar (PAB* [Penassay broth] containing agar at 15 g./l. and chloramphenicol at 10 µg./ml.). After the inoculated plate was incubated at 37°C. for about 18 hours, a single colony was selected and used for inoculating 500 ml. of sterilized PAB medium with 10 µg./ml. chloramphenicol. The resultant inoculated broth was incubated at 37°C. for about 18 hours after-which the resultant *Bacillus subtilis* MI112/pHI-16 cells were ready for harvest and subsequent isolation of plasmid DNA.

30

* PAB can be obtained from Difco Laboratories, Detroit Michigan.

B. Plasmid Isolation

About 10 g. (wet wgt) of Bacillus subtilis Mill2/pHI-16 cells were first harvested by centrifugation (10 minutes, 4°C., 10,000 rpm), then washed in
5 about 50 ml. TES, and finally collected again by centrifugation. About 20 ml. TE buffer with 25% sucrose were added to the pellet followed by about 10 mg. of lysozyme in 250 µl. water. The mixture was then incubated at 37°C. for about 30 minutes after which about
10 100 units of RNase were added. The resultant mixture was incubated at 37°C. for 30 minutes and then, upon being made 1% and 1M with respect to SDS (sodium dodecyl sulfate) and sodium chloride respectively, the mixture was cooled in an ice bath for about 3 hours.
15 After the lysate was centrifuged (30 minutes, 4°C., 19,000 rpm), the supernatant was adjusted to 31.8 ml. with TE, followed by addition of 28.7 g. of cesium chloride and .4 ml. (10 mg./ml.) of ethidium bromide. A cesium chloride gradient was established by centrifuging at 49,500 rpm for 16 hours. The plasmid band
20 was collected and centrifuged at 55,000 rpm for 16 hours, then collected again, extracted thrice with equal volumes of isoamyl alcohol, dialyzed against dilute TE, ethanol precipitated, and resuspended in
25 400 µl. of TE. The resultant plasmid pHI-16 DNA was stored at 4°C. for future use.

The kanamycin resistance gene is contained within the ~.74 kb HpaII fragment of plasmid pHI-16. Therefore, treatment with HpaII restriction enzyme
30 followed by ligation results in a ~3.9 kb plasmid,

designated herein as pHI-18, which lacks the kanamycin resistance gene. A detailed procedure for constructing plasmid pHI-18 is described below.

Example 17

5 Construction of Plasmid pHI-18

A. Partial HpaII Digestion of Plasmid pHI-16

About 5 μ l. (2.5 μ g.) of plasmid pHI-16 DNA,
1 μ l. (2 mg./ml.) BSA, 37 μ l. water, 2 μ l. of HpaII
10 (containing 2 New England Bio Labs units) restriction
enzyme, and 5 μ l. reaction mix* were incubated at 37°C.
for 1 hour. After the reaction was terminated by
heating at 65°C. for 10 minutes, the DNA was precipi-
tated by adding 2 volumes of 95% ethanol. The resul-
15 tant DNA precipitate was washed in 70% ethanol, dried in
vacuo, suspended in 5 μ l. of TE buffer, and stored at
4°C. for future use.

* Reaction mix for HpaII restriction enzyme was prepared
20 with the following composition.

60mM KCl
100mM Tris-HCl, pH 7.4
100mM MgCl₂
10mM Dithiothreitol

25 B. Ligation of Plasmid pHI-16 HpaII Digest

About 5 μ l. of plasmid pHI-16 HpaII digest
(prepared in Example 17A), 2 μ l. T4 DNA ligase, and
43 μ l. ligation mix* were incubated at about 16°C. for
30 about 18 hours. The reaction was terminated by the

addition of about 5 μ l. of 3M sodium acetate and 150 μ l. of 95% ethanol. The desired DNA precipitate was washed in 70% ethanol, dried in vacuo, suspended in 10 μ l. of TE buffer, and stored at 4°C. for future use.

5

* Ligation mix was prepared with the following composition.

66 mM Tris-HCl, pH 7.8

10 mM Dithiothreitol

10

6.6 mM $MgCl_2$

4 mM ATP

Example 18

Construction of *Bacillus subtilis* MI112/pHI-18

15

Bacillus subtilis MI112 can be obtained by conventionally culturing *B. subtilis* MI112/pHI-16 (NRRL B-12597) in the absence of chloramphenicol. The *B. subtilis* MI112/pHI-16 cells spontaneously lose the pHI-16 plasmid under the aforementioned culture conditions thus generating the desired chloramphenicol sensitive *B. subtilis* MI112 strain. Those skilled in the art will recognize and understand that sensitivity to chloramphenicol can be employed for testing and insuring that only *B. subtilis* MI112 cells that lack the plasmid are selected and used in the *Bacillus* transformation procedures herein disclosed.

25

30

About 50 ml. of sterile PAB was inoculated with *Bacillus subtilis* MI112 and incubated at 37°C. until a cell density of 2×10^8 cells/ml. was reached. The cells were then protoplasted, using sterile technique, by pelleting and then resuspending the cells in

about 5 ml. of SMMP (equal volumes of each of 4x PAB and a solution comprising 1.0M sucrose, .04M maleic acid, and .04M MgCl_2 , pH adjusted to 6.5 with NaOH). Next, about 250 μl . of lysozyme (20 mg./ml. in SMM
5 [0.5M sucrose, .02M maleic acid, and .02M MgCl_2 , pH adjusted to 6.5 with NaOH]) were added using filter sterilization. The cells were incubated with gentle shaking at 37°C. for about 2 hours. The resultant protoplasts were pelleted, washed with 5 ml. SMMP, and then resuspended in 5 ml. SMMP. Following centrifuga-
10 tion (25°C., 12 minutes, 2,600 rpm), about .1 ml. of the protoplasts were transformed by adding about 20 μl . of a 1:1 mixture comprising plasmid PHI-18 DNA (prepared in Example 17) and 2X SMM. About 1.5 ml. of PEG solution (40 g. PEG 6000 [polyethyleneglycol], 50 ml.
15 2X SMM, and water to 100 ml.) were then immediately added and, after about 2 minutes, 5 ml. of SMMP were also added. Next, the protoplasts were pelleted, suspended in 1 ml. of SMMP, and incubated at 30°C. with gentle shaking for about 2 hours. Aliquots of the thus
20 prepared suspension were plated on chloramphenicol containing DM3 regeneration medium which per liter had the following composition.

	91 g.	D-mannitol in 555 ml. deionized water containing 12 g. agar
25	10%	Casamino acids 50 ml.
	10%	Yeast extract 50 ml.
	20%	Glucose 25 ml.
	5%	Dipotassium phosphate 100 ml.
	1M	MgCl_2 20 ml.
30	10%	Gelatin 200 ml.
	10 mg	Chloramphenicol

The D-mannitol, casamino acids and yeast extract were autoclaved together. The gelatin was added immediately after autoclaving and the remaining ingredients were added after the mixture had cooled. The medium had a final chloramphenicol concentration of 10 µg./ml.

5

The resultant chloramphenicol resistant colonies were tested for kanamycin sensitivity. A chloramphenicol resistant and kanamycin sensitive colony was selected as the desired Bacillus subtilis Mill2/pHI-18 strain. The strain was cultured and the identity further confirmed by conventional restriction enzyme and gel electrophoretic analysis of the constitutive plasmid.

10

Example 19

15 Construction of Plasmids pBS1 and pBS3

A. Partial BamHI Digestion of Plasmid pEL105

About 10 µl. (5 µg.) of plasmid pEL105 (conventionally isolated from Streptomyces ambofaciens/pEL105 [prepared in Example 7] in substantial accordance with the teaching of Example 1), 2 µl. BSA (1 mg./ml.), 29 µl. water, 1 µl. of BamHI (diluted 1:4 with water) restriction enzyme, and 5 µl. reaction mix* were incubated at 25°C. for 15 minutes. The reaction was terminated by the addition of about 50 µl. of 4M ammonium acetate and 300 µl. of 95% ethanol. After cooling at -20°C. for about 2 hours, the resultant DNA precipitate was collected by centrifugation, washed twice in 70% ethanol, dried in vacuo, and then suspended

20

25

30

in about 10 μ l. of TE buffer. Because plasmid pEL105 has two BamHI restriction sites, a mixture of different fragments results.

- 5 * Reaction mix for BamHI restriction enzyme was prepared with the following composition.

1.5M NaCl
60mM Tris-HCl, pH 7.9
60mM MgCl₂

10 B. BamHI Digestion of Plasmid pHI-18

 About 5 μ l. (5 μ g.) of plasmid pHI-18, 2 μ l. BSA (1 mg./ml.), 9 μ l. water, 1 μ l. of BamHI (4 units/ μ l.) restriction enzyme, and 1.5 μ l. reaction mix were incubated at 37°C. for about 2 hours. After adding an
15 equal volume of 4M ammonium acetate and 2.5 volumes of 95% ethanol, the mixture was cooled at -20°C. for about 18 hours to precipitate the DNA. The DNA precipitate was collected by centrifugation, washed in 70% ethanol, and then suspended in about 10 μ l. of TE buffer.

20 C. Ligation

 About 5 μ l. of BamHI digested plasmid pHI-18 (prepared in Example 18B), 8 μ l. of plasmid pEL105 BamHI partial digest (prepared in Example 18A), 27 μ l. water, 5 μ l. (4mM) ATP, 5 μ l. ligation mix, and 2 μ l.
25 T4 DNA ligase were incubated at 16°C. for about 18 hours. The reaction was terminated by the addition of 50 μ l. 4M ammonium acetate and 200 μ l. of 95% ethanol. After incubation at -20°C. for about 2 hours, the
30 desired plasmid pBS1 and pBS3 DNA precipitate was

collected by centrifugation, washed in 70% ethanol, dried in vacuo, suspended in 10 μ l. of TE buffer, and stored at 4°C. for future use.

5 Since plasmid pEL105 has two BamHI restriction sites, a partial BamHI digest results in two ~4.4 kb BamHI fragments. Therefore, the insertional isomers of plasmids pBS1 and pBS3 are also produced by the above procedure. Recombinant plasmids of two orientations result because the BamHI restricted DNA can be ligated
10 in either direction. A restriction site and functional map of each of plasmids pBS1 and pBS3 is presented in Figure 6 of the accompanying drawings.

Example 20

Construction of Plasmids pBS2 and pBS4

15

The desired constructions are made in substantial accordance with the teaching of Example 19A-C except that BamHI digested plasmid pHI-16, rather than plasmid pHI-18, is used.

20 Since plasmid pEL105 has two BamHI restriction sites, a partial BamHI digest results in two ~4.4 kb BamHI fragments. Therefore, the insertional isomers of plasmids pBS2 and pBS4 are also produced by the above procedure. Recombinant plasmids of two orientations result because the BamHI restricted DNA can be ligated
25 in either direction. A restriction site and functional map of each of plasmids pBS2 and pBS4 is presented in Figure 7 of the accompanying drawings.

Example 21Construction of Plasmids pBS5 and pBS6

5 The desired constructions are made in substantial accordance with the teaching of Example 19A-C except that plasmids pEL110 and pHI-16, rather than plasmids pEL105 and pHI-18, are used. Plasmid pEL110 is conventionally isolated from Streptomyces ambofaciens/pEL110 (prepared in Example 9) in substantial accordance with the teaching of Example 1.

10 Since plasmid pEL110 has two BamHI restriction sites, a partial BamHI digest results in two ~6.2 kb BamHI fragments. Therefore, the insertional isomers of plasmids pBS5 and pBS6 are also produced by the above procedure. Recombinant plasmids of two orientations
15 result because the BamHI restricted DNA can be ligated in either direction. A restriction site and functional map of each of plasmids pBS5 and pBS6 is presented in Figure 13 of the accompanying drawings.

Example 22

20

Construction of Plasmids pBS7 and pBS8

 The desired constructions are made in substantial accordance with the teaching of Example 19A-C except that plasmids pEL113 and pHI-16, rather than
25 plasmids pEL105 and pHI-18, are used. Plasmid pEL113 is conventionally isolated from Streptomyces ambofaciens/pEL113 (prepared in Example 11) in substantial accordance with the teaching of Example 1.

 Since plasmid pEL113 has three BamHI re-
30 striction sites, a partial BamHI digest results in

three ~7.8 kb BamHI fragments. Therefore, the insertional isomers of plasmids pBS7 and pBS8 are also produced by the above procedure. Recombinant plasmids of two orientations result because the BamHI restricted DNA can be ligated in either direction. A restriction site and functional map of each of plasmids pBS7 and pBS8 is presented in Figure 14 of the accompanying drawings.

Example 23

10 Construction of Plasmids pBS9 and pBS10

The desired constructions are made in substantial accordance with the teaching of Example 19A-C except that plasmids pEL122 and pHI-16, rather than plasmids pEL105 and pHI-18, are used. Plasmid pEL122 is isolated from E. coli K12 HB101/pEL122 (prepared in Example 15) according to known procedures.

Since plasmid pEL122 has three BamHI restriction sites, a partial BamHI digest results in three ~8.7 kb BamHI fragments. Therefore, the insertional isomers of plasmids pBS9 and pBS10 are also produced by the above procedure. Recombinant plasmids of two orientations result because the BamHI restricted DNA can be ligated in either direction. A restriction site and functional map of each of plasmids pBS9 and pBS10 is presented in Figure 15 of the accompanying drawings.

Example 24

Construction of Bacillus subtilis MI112/pBS1 and

30 Bacillus subtilis MI112/pBS3

The desired constructions are made in substantial accordance with the teaching of Example 18

except that DNA comprising plasmids pBS1 and pBS3 (prepared in Example 19), rather than plasmid pHI-18, is used. The resultant Bacillus subtilis MI112/pBS1 and Bacillus subtilis MI112/pBS3 chloramphenicol resistant and kanamycin sensitive transformant colonies are isolated according to known procedures, cultured, and then conventionally identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

10

Example 25Construction of Bacillus subtilis MI112/pBS2 and Bacillus subtilis MI112/pBS4

The desired constructions are made in substantial accordance with the teaching of Example 18 except that DNA comprising plasmids pBS2 and pBS4 (prepared in Example 20), rather than plasmid pHI-18, is used. The resultant chloramphenicol resistant colonies were selected and conventionally tested for kanamycin resistance. The chloramphenicol and kanamycin resistant Bacillus subtilis MI112/pBS2 and B. subtilis MI112/pBS4 transformants are isolated according to known procedures, cultured, and then conventionally identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

25

Example 26Construction of Bacillus subtilis MI112/pBS5 and Bacillus subtilis MI112/pBS6

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The desired constructions are made in substantial accordance with the teaching of Example 18

except that DNA comprising plasmids pBS5 and pBS6 (prepared in Example 21), rather than plasmid pHI-18, is used. The resultant chloramphenicol resistant colonies are selected and conventionally tested for kanamycin resistance. The chloramphenicol and kanamycin resistant Bacillus subtilis M112/pBS5 and B. subtilis M112/pBS6 transformants are isolated according to known procedures, cultured, and then conventionally identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

Example 27

Construction of Bacillus subtilis M112/pBS7 and Bacillus subtilis M112/pBS8

The desired constructions are made in substantial accordance with the teaching of Example 18 except that DNA comprising plasmids pBS7 and pBS8 (prepared in Example 22), rather than plasmid pHI-18, is used. The resultant chloramphenicol resistant colonies are selected and conventionally tested for kanamycin resistance. The chloramphenicol and kanamycin resistant Bacillus subtilis M112/pBS7 and B. subtilis M112/pBS8 transformants are selected according to known procedures, cultured, and then conventionally identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

Example 28Construction of *Bacillus subtilis* M112/pBS9 and
Bacillus subtilis M112/pBS10

5 The desired constructions are made in substantial accordance with the teaching of Example 18 except that DNA comprising plasmids pBS9 and pBS10 (prepared in Example 23), rather than plasmid pHI-18, is used. The resultant chloramphenicol resistant colonies
10 are selected and conventionally tested for kanamycin resistance. The putative chloramphenicol and kanamycin resistant *Bacillus subtilis* M112/pBS9 and *B. subtilis* M112/pBS10 transformants are selected according to known procedures, cultured, and then conventionally
15 identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

Example 29Construction of *Streptomyces ambofaciens*/pBS1 and
20 *S. ambofaciens*/pBS3

 Using about 20 µg. of the DNA from Example 19 and 1×10^8 protoplasts of *Streptomyces ambofaciens* (NRRL No. 2420), the desired constructions are made in
25 substantial accordance with the teaching of International Publication (of International Patent Application No. PCT/GB79/00095) No. W079/01169, Example 2. The desired transformants are selected for thiostrepton resistance by overlaying the regenerating protoplasts with modified R2 medium top agar containing sufficient
30 thiostrepton to bring the final plate concentration to 50 µg./ml.

The resultant Streptomyces ambofaciens/pBS1 and S. ambofaciens/pBS3 thiostrepton resistant colonies are isolated according to known procedures, cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids in substantial accordance with the teaching of Example 7.

Example 30

Construction of Streptomyces ambofaciens/pBS2 and S. ambofaciens/pBS4

Using about 20 μ g. of the DNA from Example 20 and 1×10^8 protoplasts of Streptomyces ambofaciens (NRRL No. 2420), the desired constructions are made in substantial accordance with the teaching of International Publication (of International Patent Application No. PCT/GB79/00095) No. W079/01169, Example 2. The desired transformants are selected for thiostrepton resistance by overlaying the regenerating protoplasts with modified R2 medium top agar containing sufficient thiostrepton to bring the final plate concentration to 50 μ g./ml.

The resultant Streptomyces ambofaciens/pBS2 resistant colonies are isolated according to known procedures, cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids in substantial accordance with the teaching of Example 7.

Example 31Construction of *Streptomyces ambofaciens*/pBS5 and
S. ambofaciens/pBS6

- 5 Using about 20 µg. of the DNA from Example 21
and 1×10^8 protoplasts of *Streptomyces ambofaciens* (NRRL
No. 2420), the desired constructions are made in
substantial accordance with the teaching of International
10 PCT/GB79/00095) No. W079/01169, Example 2. The desired
transformants are selected for neomycin resistance by
overlaying the regenerating protoplasts with modified
R2 medium top agar containing sufficient neomycin to
bring the final plate concentration to 1 µg./ml.
- 15 The resultant *Streptomyces ambofaciens*/pBS5
and *S. ambofaciens*/pBS6 neomycin resistant colonies are
isolated according to known procedures, cultured, and
then identified by restriction enzyme and gel electro-
phoretic analysis of the constitutive plasmids in
20 substantial accordance with Example 9.

Example 32Construction of *Streptomyces ambofaciens*/pBS7 and
S. ambofaciens/pBS8

- 25 Using about 20 µg. of the DNA from Example 22
and 1×10^8 protoplasts of *Streptomyces ambofaciens* (NRRL
No. 2420), the desired constructions are made in
substantial accordance with the teaching of International
30 Publication (of International Patent Application No.

PCT/GB79/00095) No. W079/01169, Example 2. The desired transformants are selected first for thiostrepton resistance and then for neomycin resistance by the methods described in Examples 7 and 9 above.

5 The resultant Streptomyces ambofaciens/pBS7 and S. ambofaciens/pBS8 thiostrepton and neomycin resistant colonies are isolated according to known procedures, cultured, and then identified by restriction enzyme and gel electrophoretic analysis of the con-
10 stitutive plasmids in substantial accordance with the teaching of Example 11.

Example 33

Construction of Streptomyces ambofaciens/pBS9 and
15 S. ambofaciens/pBS10

 Using about 20 µg. of the DNA from Example 23 and 1×10^8 protoplasts of Streptomyces ambofaciens (NRRL No. 2420), the desired constructions are made in
20 substantial accordance with the teaching of International Publication (of International Patent Application No. PCT/GB79/00095) No. W079/01169, Example 2. The desired transformants are selected for thiostrepton resistance by overlaying the regenerating protoplasts
25 with modified R2 medium top agar containing sufficient thiostrepton to bring the final plate concentration to 50 µg./ml.

 The resultant Streptomyces ambofaciens/pBS9 and S. ambofaciens/pBS10 thiostrepton resistant colonies
30 are isolated according to known procedures, cultured,

and then identified by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids in substantial accordance with the teaching of Example 7.

Example 34

5 Construction of E. coli K12 HB101/pBS9 and
 E. coli K12 HB101/pBS10

 The desired constructions are made in substantial accordance with the teaching of Example 3 with
10 the exception that DNA prepared in Example 23, rather than plasmid pLR2, is used for the transformation. Surviving colonies are first selected, tested for the expected phenotype (Amp^R , Tet^S), and then conventionally identified as the desired E. coli K12 HB101/pBS9 and E.
15 coli K12 HB101/pBS10 transformants by restriction enzyme and gel electrophoretic analysis of the constitutive plasmids.

 Representative plasmids and transformants that can be constructed according to the foregoing
20 teaching are respectively shown in Tables I and II below. For purposes of the present invention, the pBS plasmids named in Table 1 also include any insertional isomers resulting from a particular construction.

25

30

Table 1Representative Plasmids

	<u>Example No.</u>	<u>Plasmid Name*</u>	<u>Construction</u>
			<u>Ligation of <u>Bam</u>HI Fragments</u>
5	35	pBS11 and pBS12	pHI-16 and pEL107
	36	pBS13 and pBS14	pHI-16 and pEL109
	37	pBS15 and pBS16	pHI-16 and pEL114
	38	pBS17 and pBS18	pHI-16 and pEL115
	39	pBS19 and pBS20	pHI-16 and pEL116
10	40	pBS21 and pBS22	pHI-16 and pEL121
	41	pBS23 and pBS24	pHI-18 and pEL107
	42	pBS25 and pBS26	pHI-18 and pEL109
	43	pBS27 and pBS28	pHI-18 and pEL110
	44	pBS29 and pBS30	pHI-18 and pEL113
15	45	pBS31 and pBS32	pHI-18 and pEL114
	46	pBS33 and pBS34	pHI-18 and pEL115
	47	pBS35 and pBS36	pHI-18 and pEL116
	48	pBS37 and pBS38	pHI-18 and pEL121
	49	pBS39 and pBS40	pHI-18 and pEL122
20	50	pBS41 and pBS42	pBR322 and pBS13
	51	pBS43 and pBS44	pBR322 and pBS18
	52	pBS45 and pBS46	pBR322 and pBS7
	53	pBS47 and pBS48	pBR322 and pBS27

25

30

Table I continued

Example No.	Plasmid Name*	Construction	
		Ligation of <u>Bam</u> HI Fragments	
5	54	pBS49 and pBS50	pBR322 and pBS2
	55	pBS51 and pBS52	pBR322 and pBS1
	56	pBS53 and pBS54	pBR328 and pBS2
	57	pBS55 and pBS56	pBR328 and pBS1
		Ligation of <u>Eco</u> RI Fragments	
10	58	pBS57 and pBS58	pBR328 and pBS2
	59	pBS59 and pBS60	pBR328 and pBS1

15 * The first named plasmid, in Examples 35-49 above, designates the orientation in which the pEL thiostrepton or neomycin resistance gene is opposite (rather than adjacent to) the pHI kanamycin or chloramphenicol resistance gene. The second plasmid designates the reverse orientation.

20 The first named plasmid, in Examples 50-57 above, designates the orientation in which the pHI PvuII site is adjacent to the pBR HindIII site. The second plasmid designates the reverse orientation.

25 The first named plasmid, in Examples 58-59 above, designates the orientation in which the pHI XbaI site is adjacent to the pBR PvuII site. The second plasmid designates the reverse orientation.

30

Table IIRepresentative Transformants

1. Bacillus R/pR^1 wherein R is subtilis, subtilis
MI112, RUB331, thuringiensis, megaterium, cereus,
5 popillae, laterosporus, or amyloliquifaciens
and R^1 independently is any pBS plasmid herein
named in Examples 35-59 of Table 1.
2. Streptomyces R^2/pR^1 wherein R^2 is ambofaciens,
10 fradiae, coelicolor, aureofaciens, granuloruber,
or lividans and R^1 independently is as defined
above.
3. E. coli R^3/pR^4 wherein R^3 is K12, K12 HB101, K12
RV308, C600 R_k-M_k- , or K12 C600 and R^4 independently
15 is any pBS plasmid herein named in Examples 48-59
cf Table 1.

20

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CLAIMS

1. A multifunctional recombinant DNA cloning vector comprising:

- 5 a) two or more functionally different origins of replication that are independently selected from the group consisting of any origin of replication that is functional in Streptomyces, any origin of replication that is functional in Bacillus, and any origin of replication that is functional in E. coli,
10 and
- b) one or more DNA segments that confer resistance to at least one antibiotic when transformed into a sensitive host cell in which an origin of replication comprising
15 said vector is functional, said host cells being susceptible to transformation, cell division, and culture,
- subject to the limitation that when said vector is limited to two functionally different origins of
20 replication, neither of the origins of replication is functional in E. coli.

2. The vector of claim 1 wherein the origins of replication that are functional in Streptomyces, Bacillus, and E. coli are respectively a restriction
25 fragment of plasmid pEL103, a restriction fragment of plasmid pHI-16, and a restriction fragment of an E. coli plasmid.

3. The vector of claim 1 or 2 wherein the origins of replication in the vector are a restriction
30 fragment of plasmid pEL103 and a restriction fragment of plasmid pHI-16.

4. The vector of claim 2 or 3 wherein the origin of replication containing restriction fragment of plasmid pEL103 is the ~2.8 kb BamHI fragment.

5. The vector of claim 2 in which the E. coli plasmid is plasmid pBR322 or pBR328.

5 6. The vector of any of claims 1 to 4 wherein the ~1.6 kb BamHI restriction fragment of plasmid pLR2 conveys resistance to thiostrepton.

7. The vector of any of claims 1 to 4 wherein the ~3.4 kb BamHI restriction fragment of
10 plasmid pLR1 conveys resistance to neomycin.

8. The vector of any of claims 1 to 4 wherein the ~4.6 kb BamHI restriction fragment of plasmid pHI-16 conveys resistance to chloramphenicol.

9. The vector of any of claims 1 to 4
15 wherein the ~3.9 kb BamHI restriction fragment of plasmid pHI-18 conveys resistance to kanamycin.

10. The vector of any of claims 1 to 9 which is plasmid pBS1, pBS2, pBS3, pBS4, pBS5, pBS6, pBS7, pBS8, pBS9, pBS10, pBS11, pBS12, pBS13, pBS14, pBS15, pBS16, pBS17, pBS18, pBS19, pBS20, pBS21, pBS22, pBS23, pBS24, pBS25, pBS26, pBS27, pBS28, pBS29, pBS30, pBS31, pBS32, pBS33, pBS34, pBS35, pBS36, pBS37, pBS38, pBS39, pBS40, pBS41, pBS42, pBS43, pBS44, pBS45, pBS46, pBS47, pBS48, pBS49, pBS50, pBS51, pBS52, pBS53, pBS54, pBS55, pBS56, pBS57, pBS58, pBS59, or pBS60.
20
25

11. The vector of claim 10 which is plasmid pBS1, pBS2, pBS3, pBS4, pBS5, pBS6, pBS7 or pBS8.

12. The vector of claim 10 which is pBS9, pBS10, pBS49 or pBS60.

13. A transformed restrictionless host cell comprising the recombinant DNA cloning vector of any of claims 1 to 11 subject to the limitation that said host cell, prior to transformation, is sensitive to an antibiotic for which resistance is conferred by a DNA segment comprising said vector, and further that at least one origin of replication comprising said vector is functional in said host cell.

14. The host cell of claim 13 which is a Streptomyces, preferably of species ambofaciens, coelicolor or fradiae.

15. The host cell of claim 13 which is a Bacillus, preferably of species subtilis.

16. The host cell of claim 13 which is an E. coli comprising plasmid pBS9, pBS49 or pBS60.

17. The host cell of claim 16 which is E. coli K12 HB101/pBS9.

18. A process for preparing a multifunctional recombinant cloning vector which comprises ligating at least two of

- 1) a restriction fragment comprising an origin of replication functional in Streptomyces and one or more DNA segments that confer resistance to at least one antibiotic when transformed into Streptomyces,
- 2) a restriction fragment comprising an origin of replication functional in Bacillus and one or more DNA segments that confer resistance to at least one antibiotic when transformed into Bacillus

- 3) a restriction fragment comprising an origin of replication functional in E. coli and one or more DNA segments that confer resistance to at least one antibiotic when transformed into E. coli
- 5 subject to the limitation that when said vector is limited to two functionally different origins of replication, neither of the origins of replication is functional in E. coli.
- 10 19. The process of claim 18 for preparing a cloning vector functional in Streptomyces, Bacillus and E. coli wherein the origins of replication in the vector are a restriction fragment of plasmid pEL103, a restriction fragment of plasmid pHI16, and a restriction fragment of plasmid pBR322.
- 15 20. A recombinant DNA cloning vector which is plasmid pHI16 or pHI18.
- 20 21. A process for isolating plasmid pHI16 which comprises lysing the cells of a culture of Bacillus subtilis MI112/pHI16 and separating plasmid from the lysate.
22. The process of claim 21 in which the cells are treated with lysozyme and the plasmid is separated by centrifugation.
- 25 23. A process for preparing plasmid pHI18 which comprises self ligating a partial HpaII digest of plasmid pHI16.

FIG. I
Restriction Site and Functional Map
of Plasmid pEL103

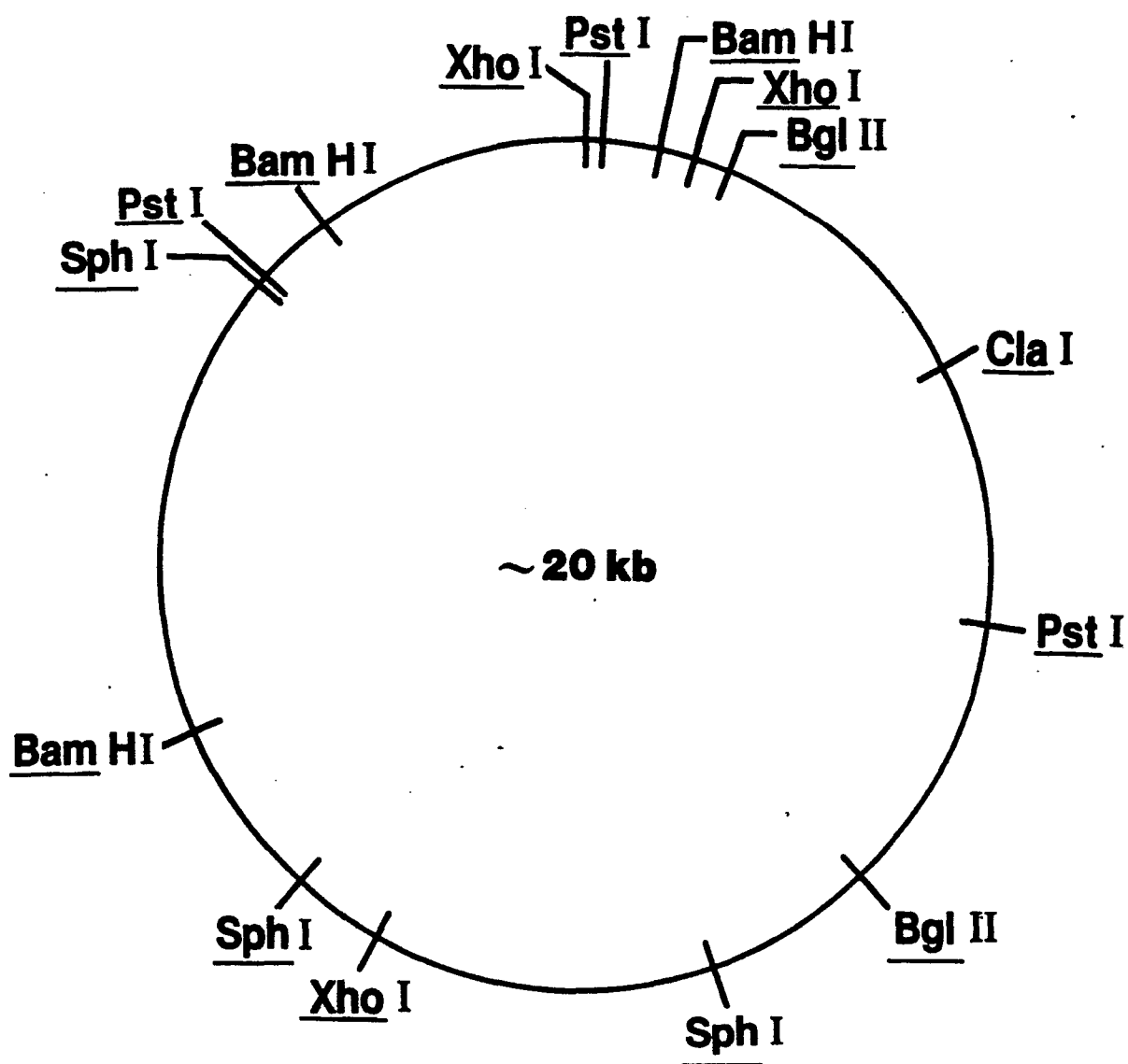


FIG. 2
Restriction Site and Functional Map
of Plasmid pLR1

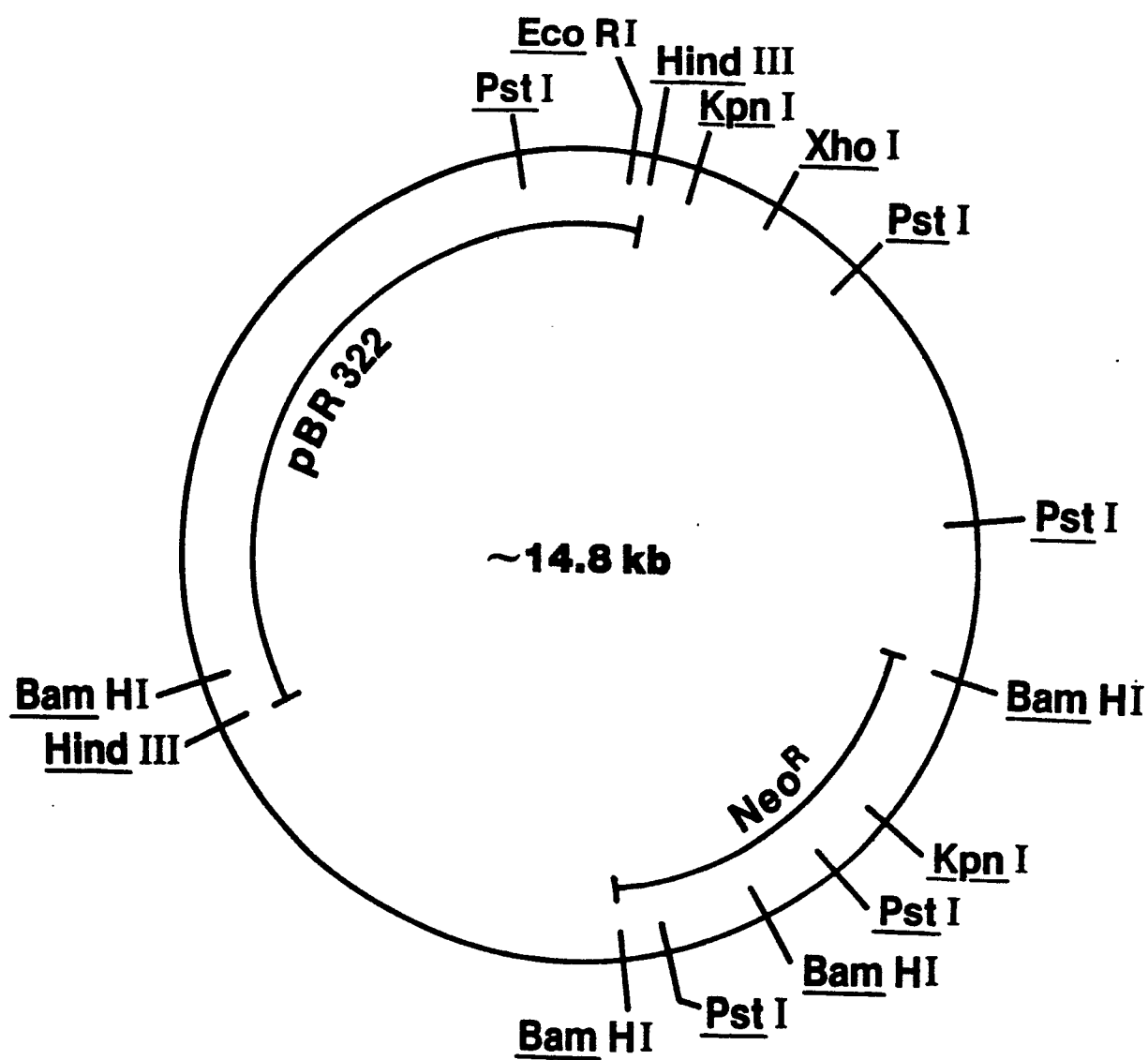


FIG. 3
Restriction Site and Functional Map
of Plasmid pLR2

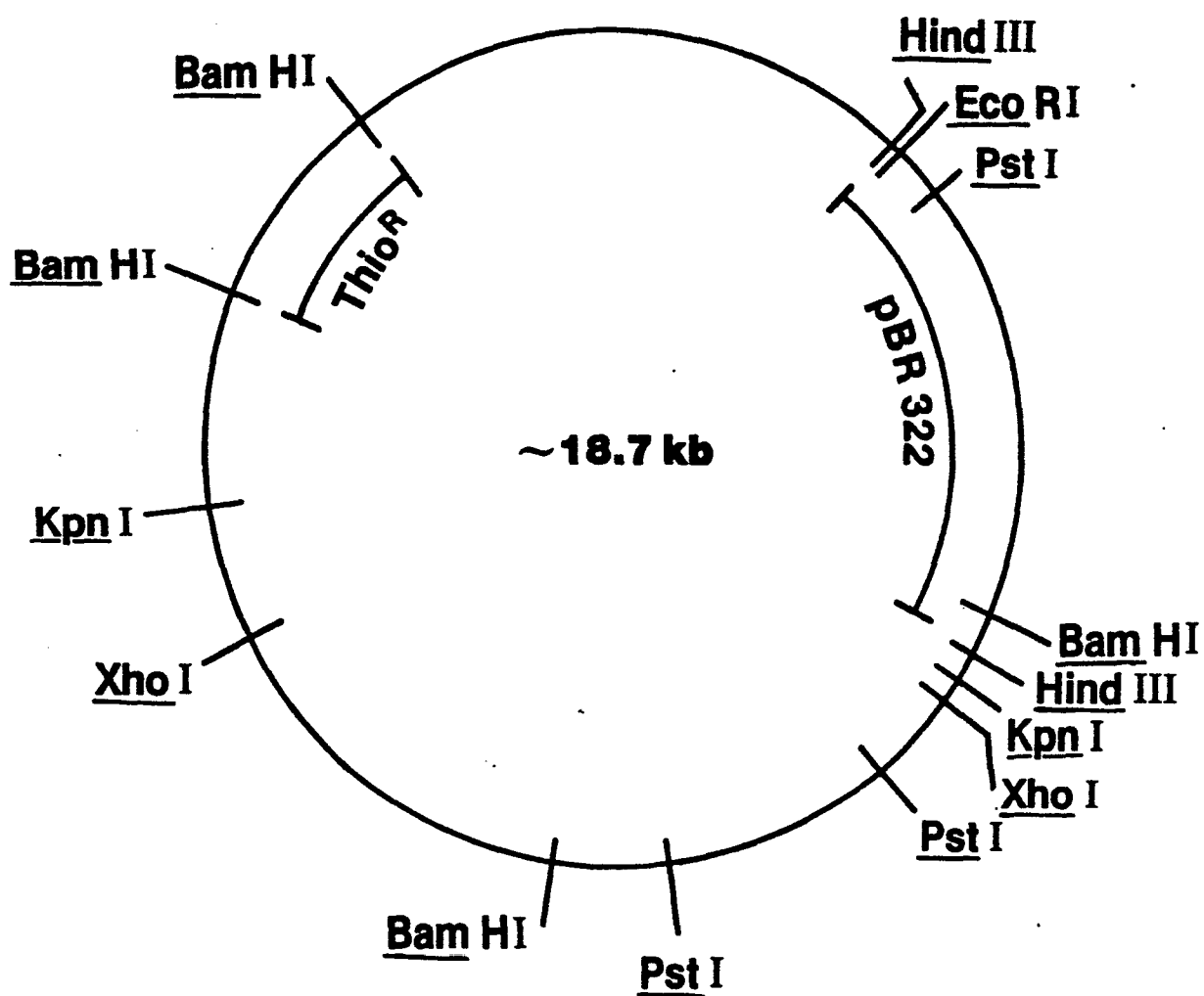


FIG. 4
Restriction Site and Functional Map
of Plasmid pHI-16

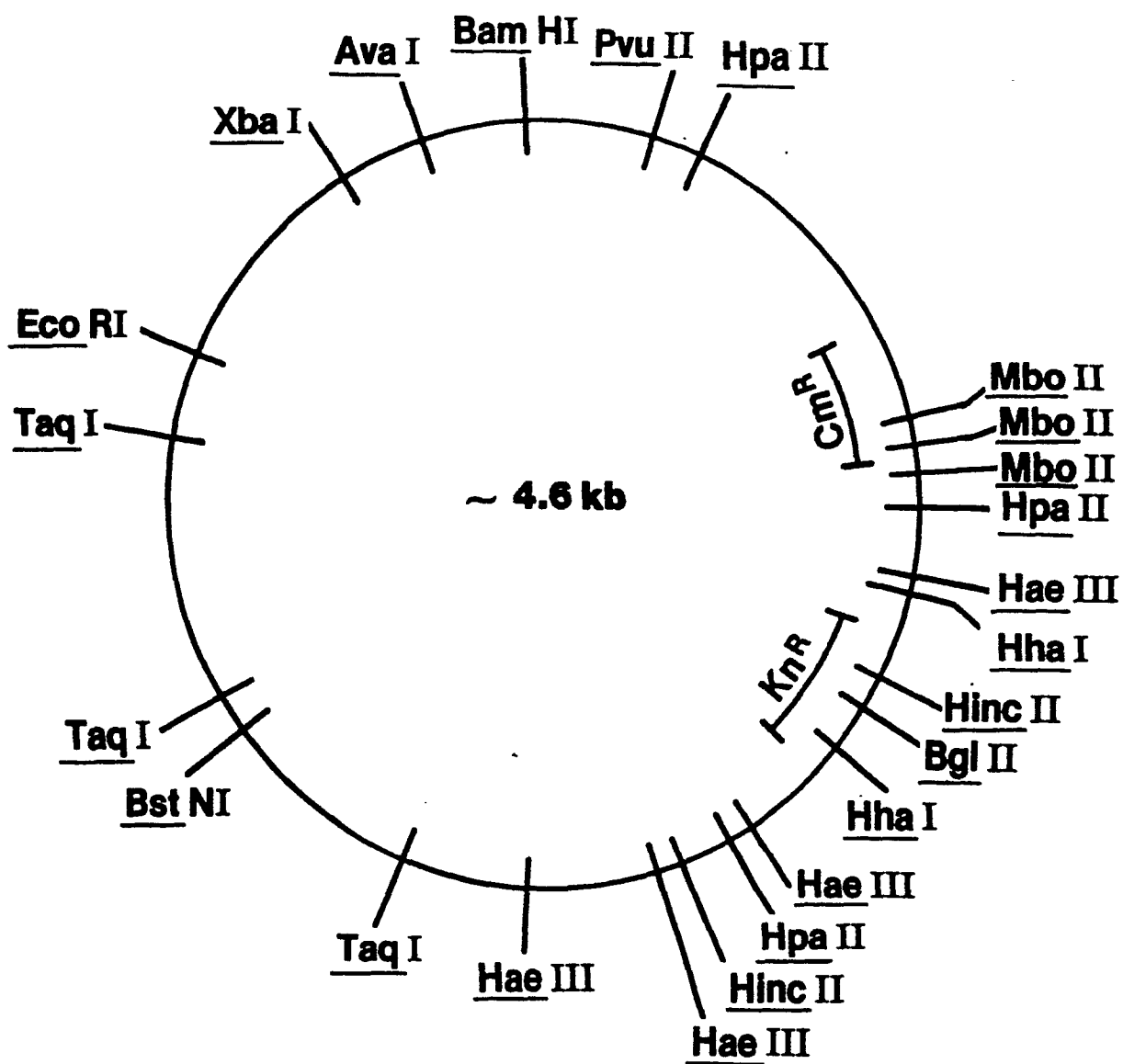
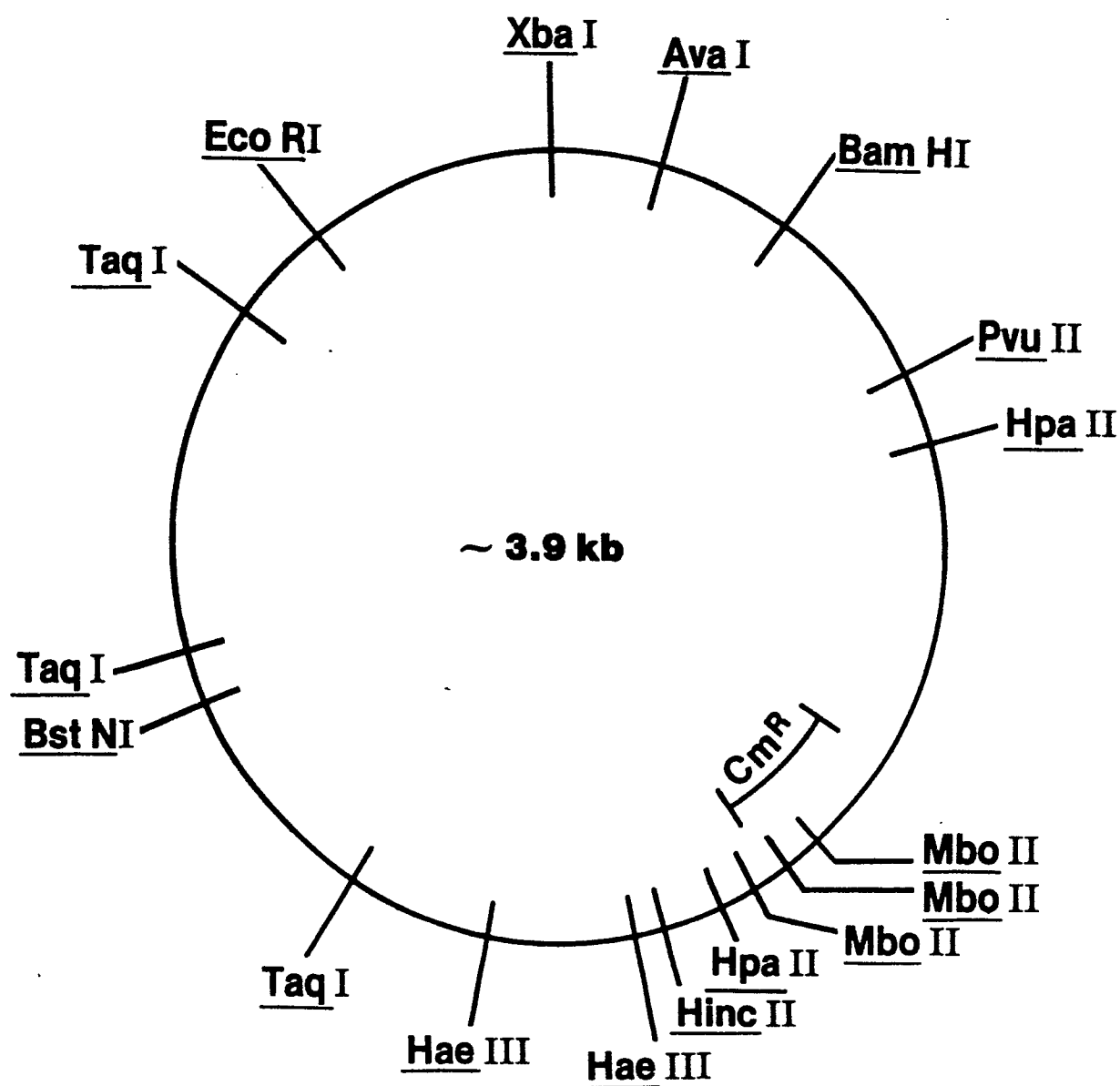


FIG. 5
Restriction Site and Functional Map
of Plasmid pH1-18



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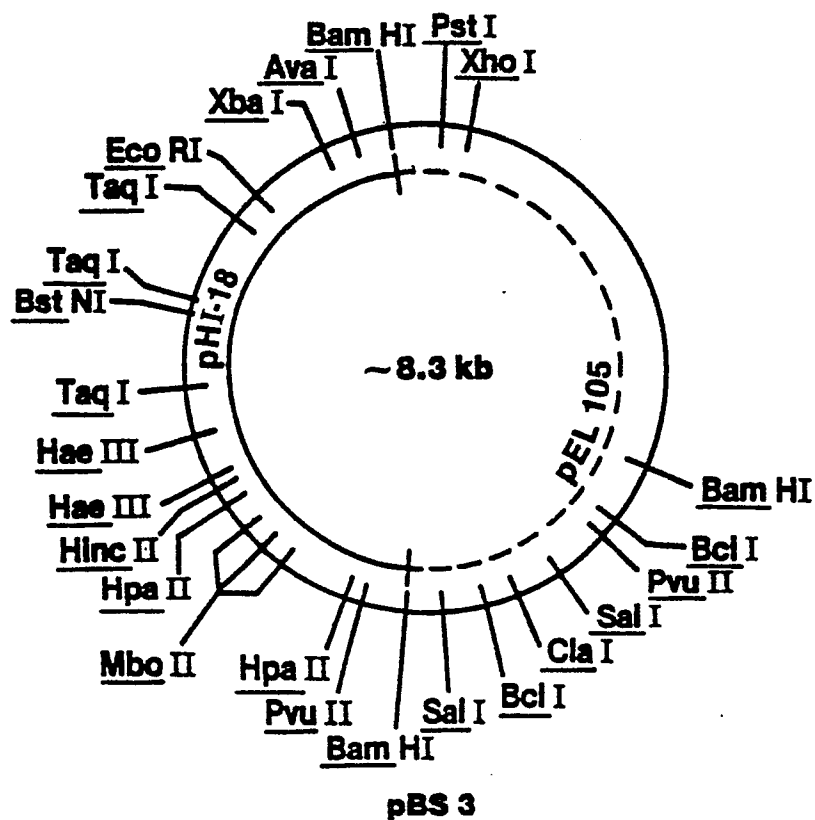
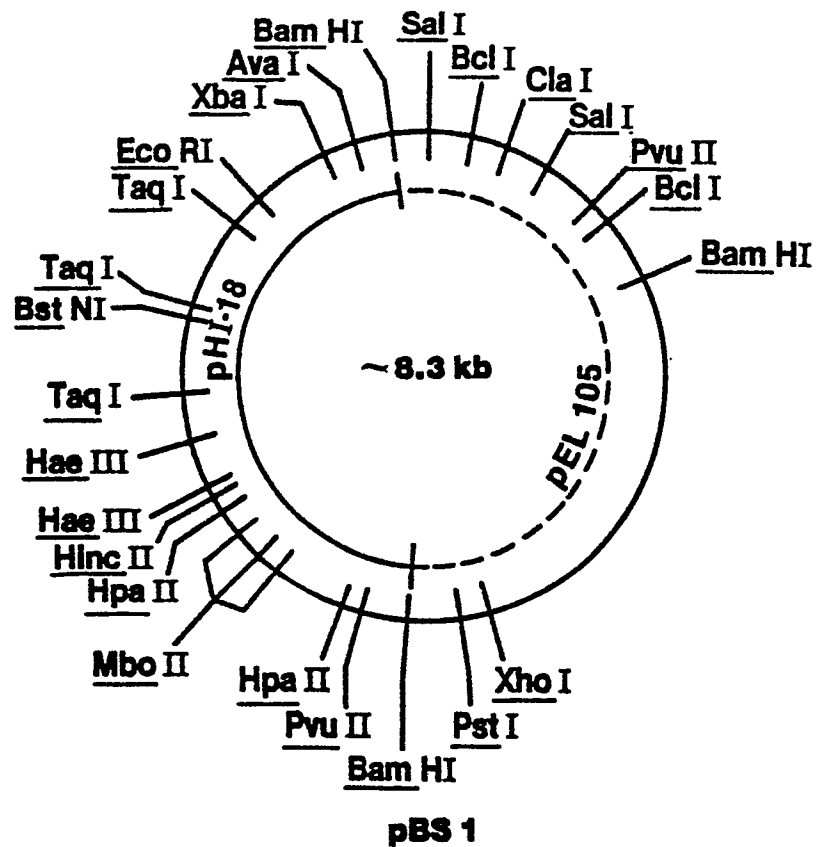
FIG. 6**Restriction Site and Functional Map
of Plasmids pBS 1 and pBS 3**

FIG. 7
Restriction Site and Functional Map
of Plasmids pBS 2 and pBS 4

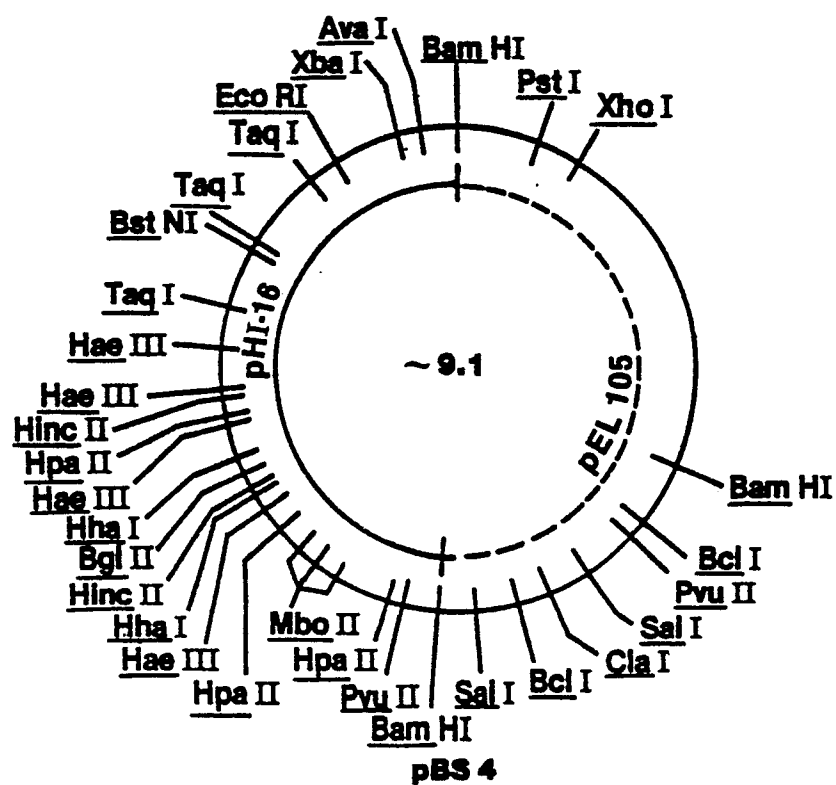
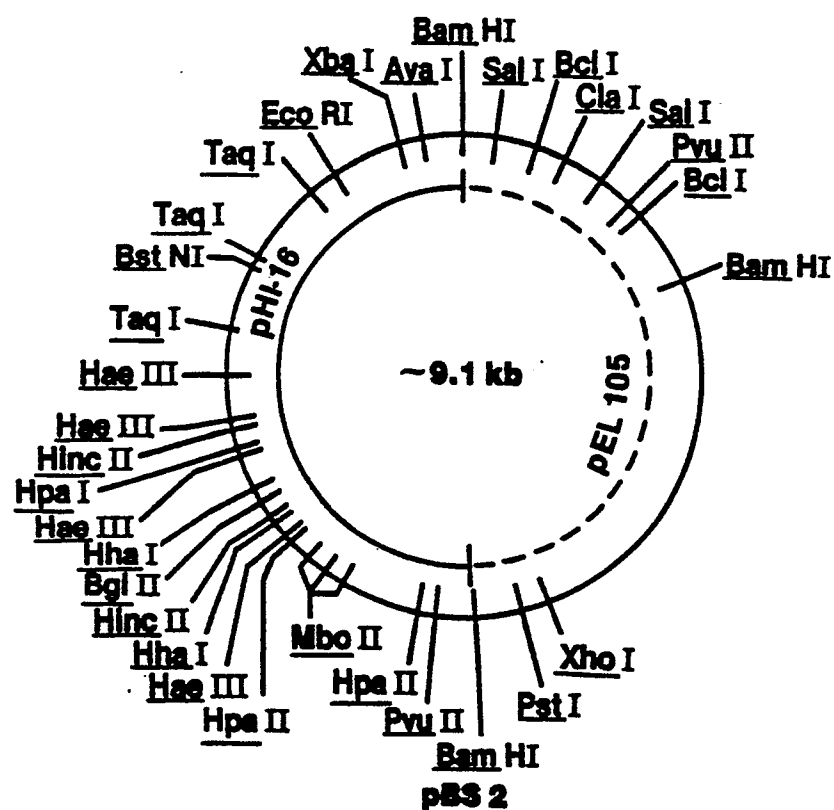


FIG. 8
Restriction Site and Functional Map
of Plasmids pEL107 and pEL105

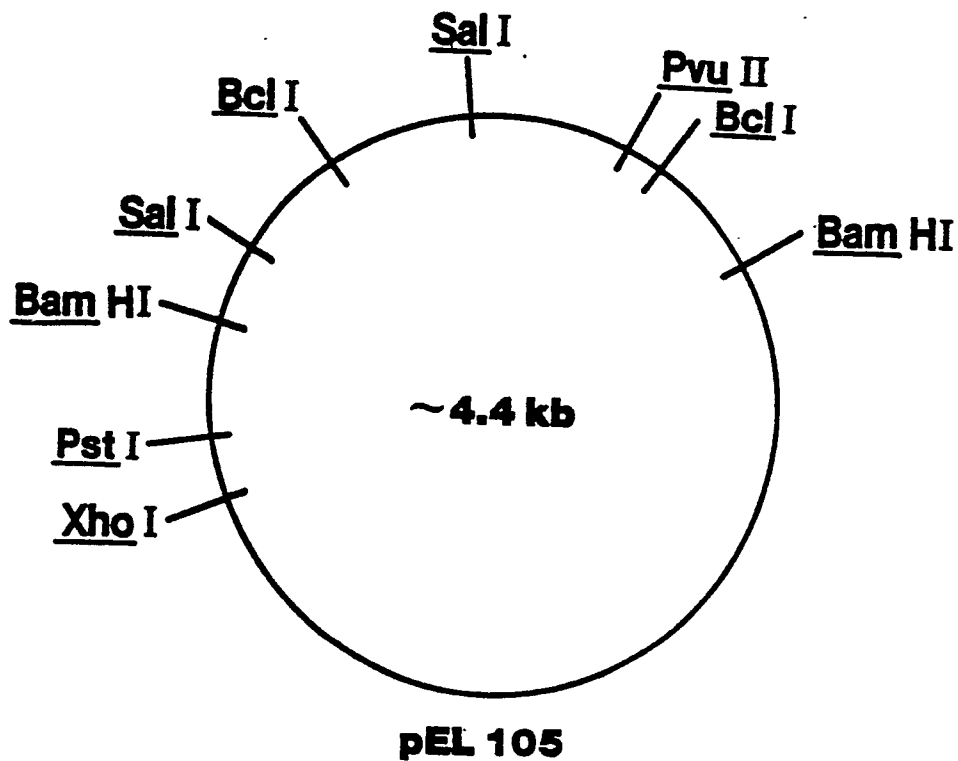
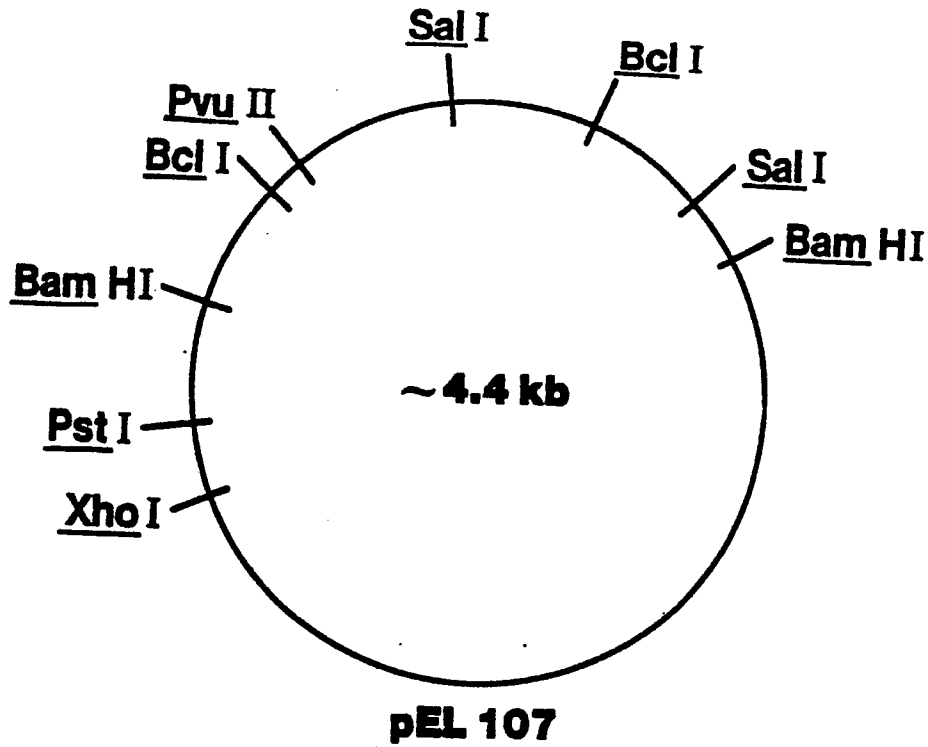
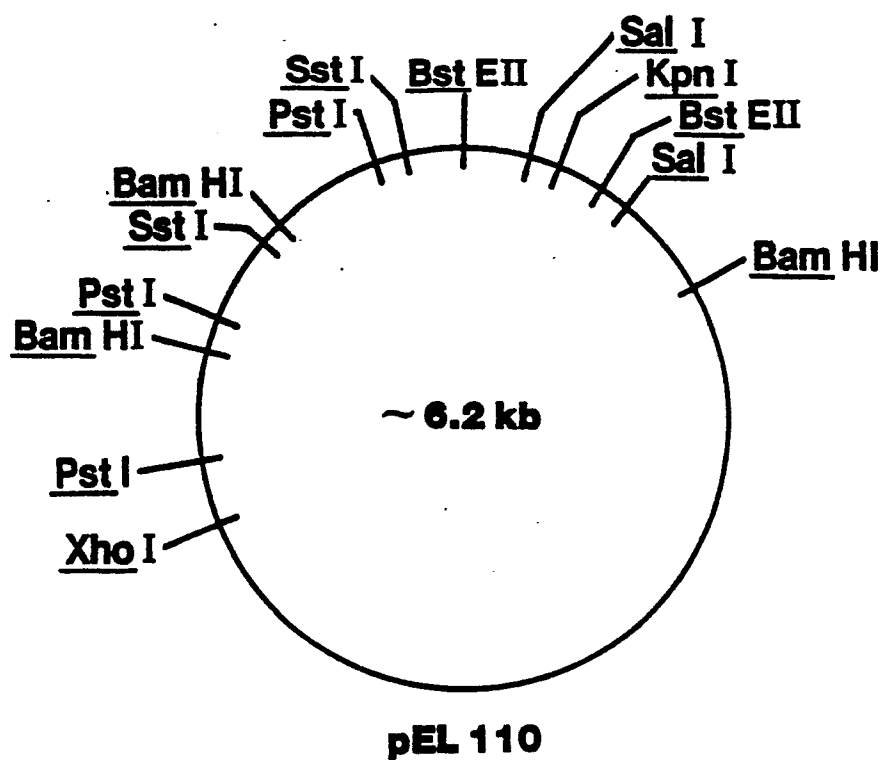
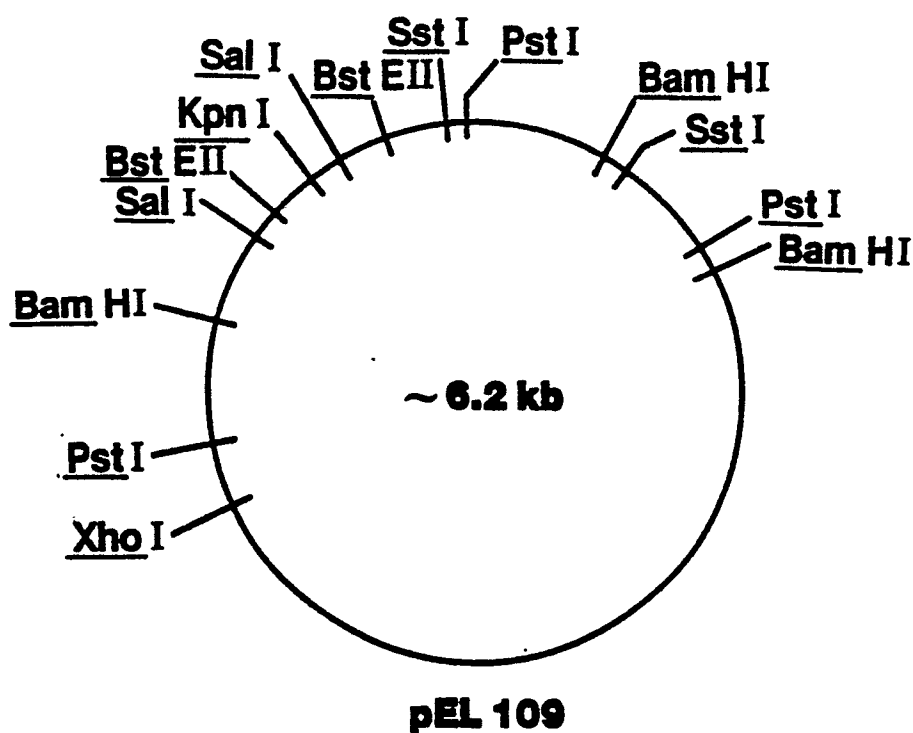
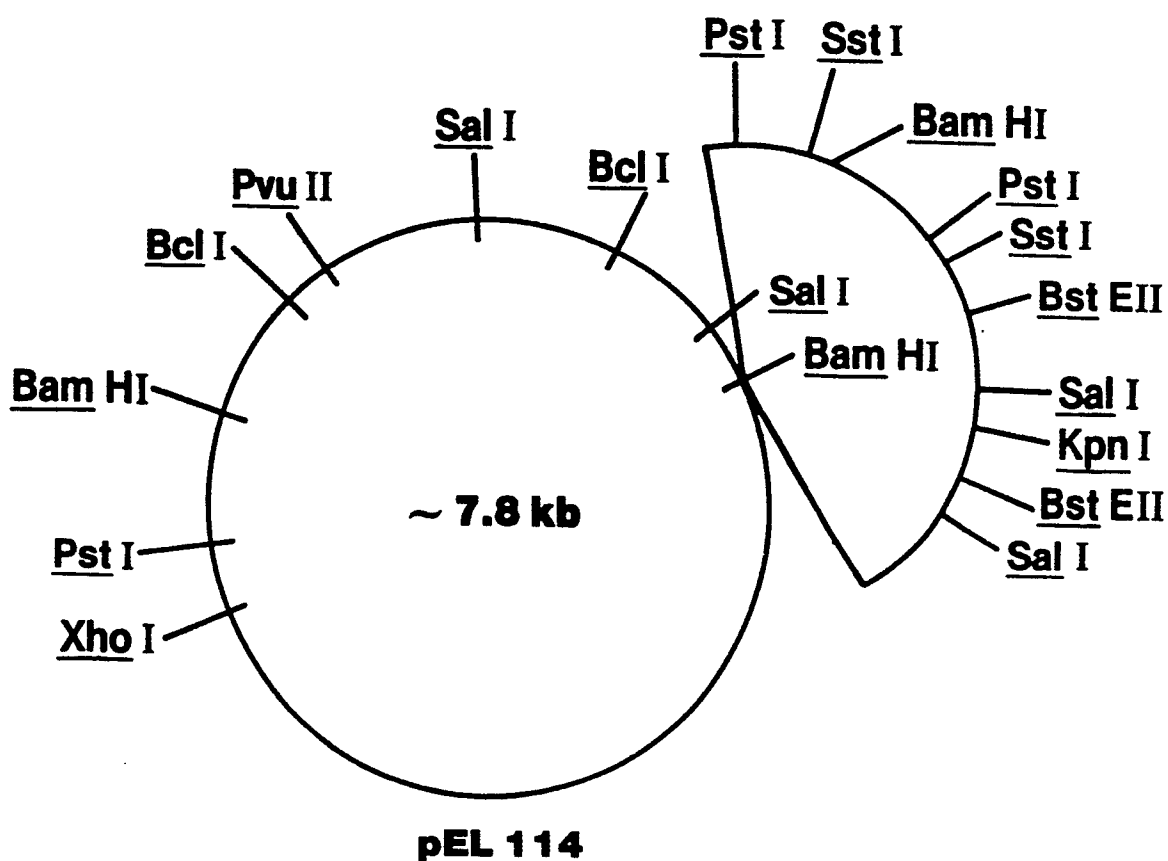
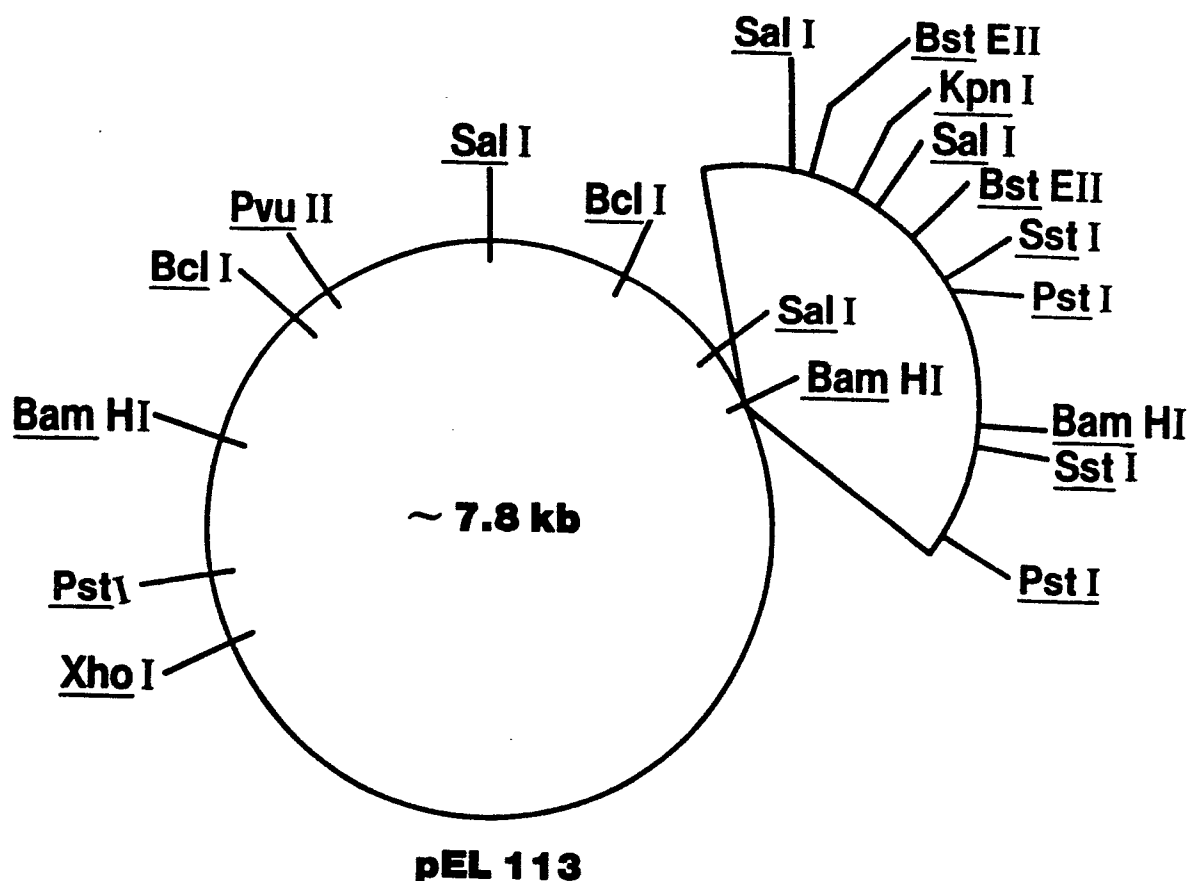


FIG. 9
Restriction Site and Functional Map
of Plasmids pEL109 and pEL110



Restriction Site and Functional Map of Plasmids pEL113 and pEL114



Restriction Site and Functional Map of Plasmids pEL115 and pEL116

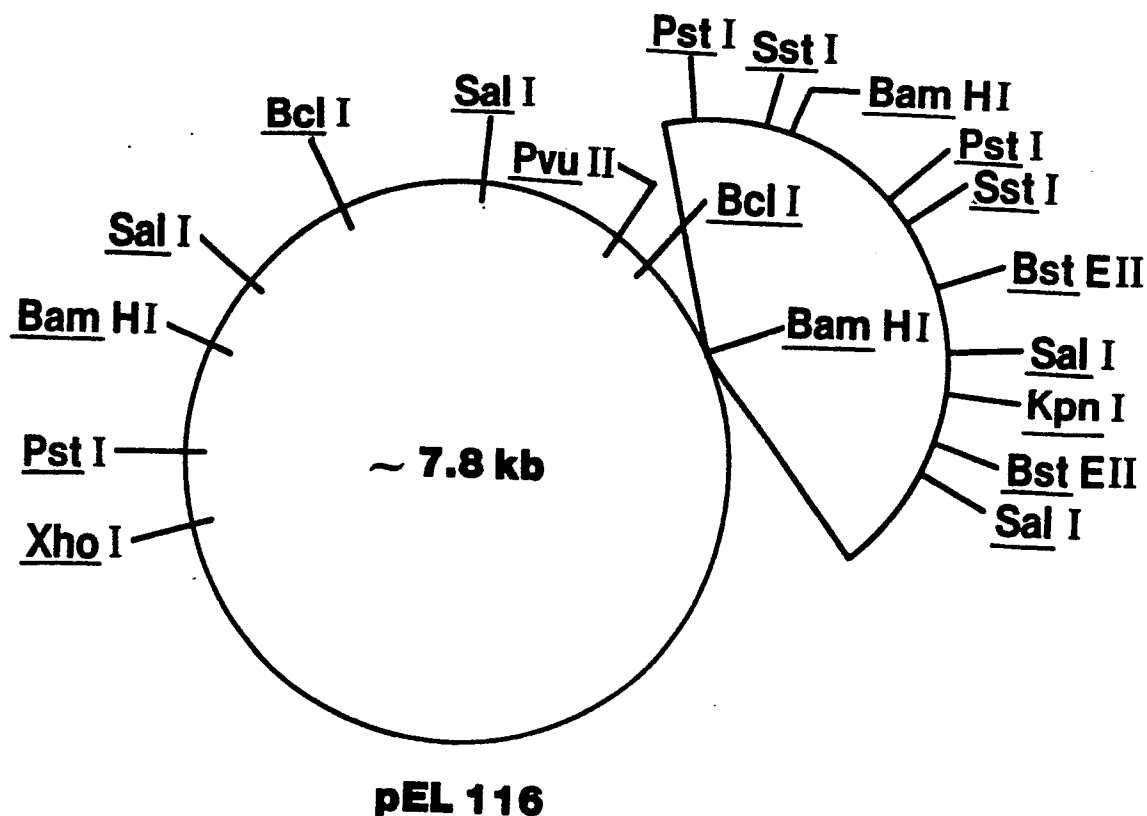
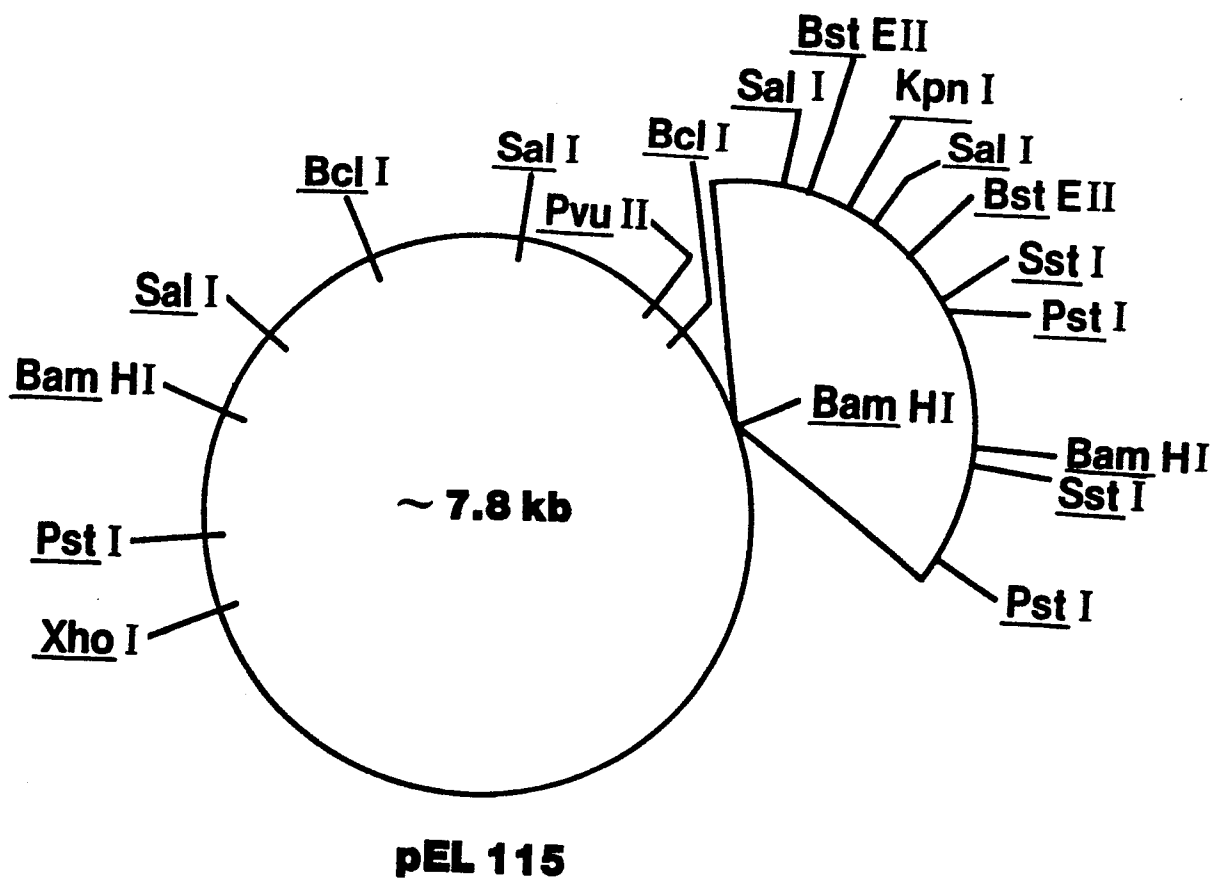


FIG. 12
Restriction Site and Functional Map
of Plasmids pEL121 and pEL122

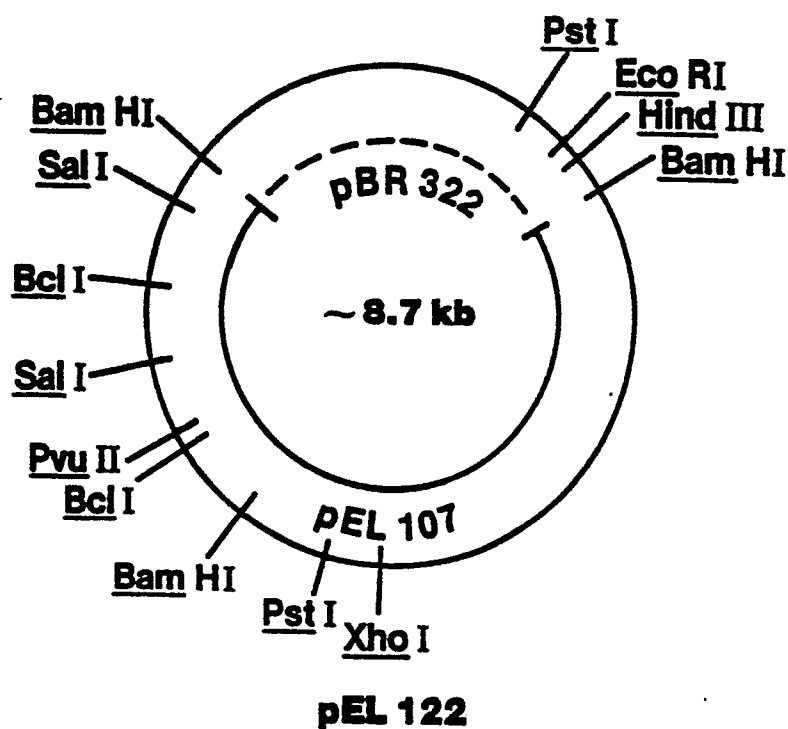
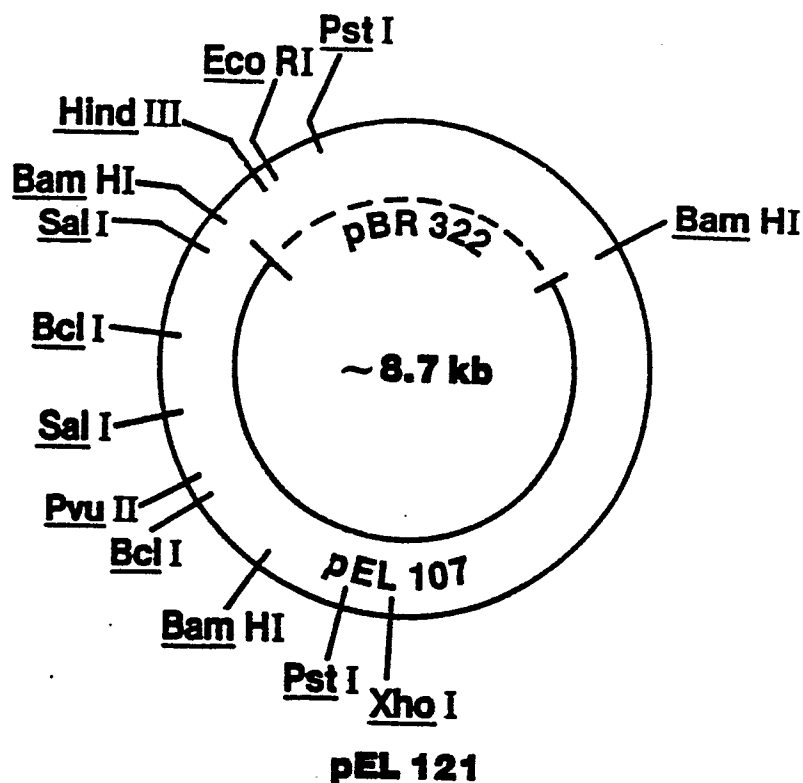


FIG. 13
Restriction Site and Functional Map
of Plasmids pBS5 and pBS6

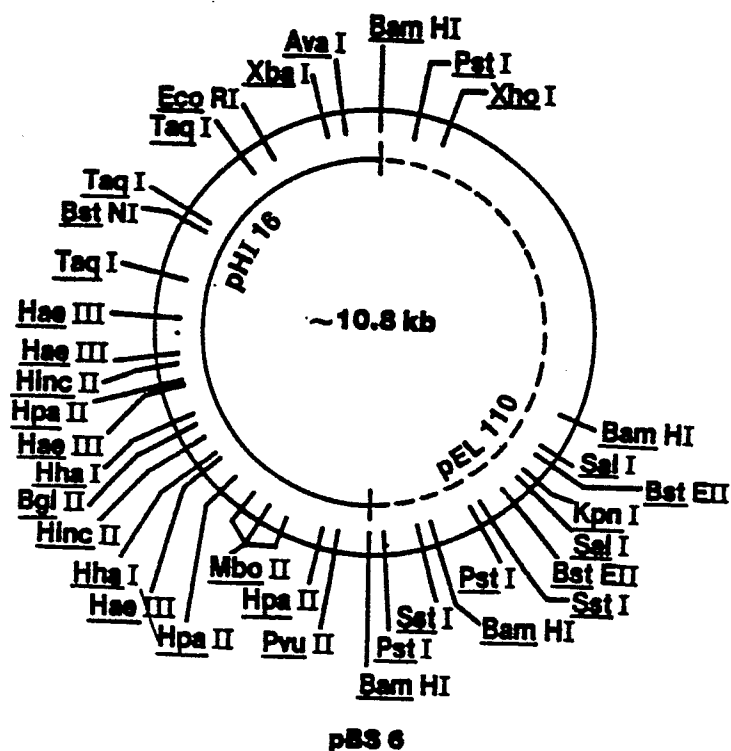
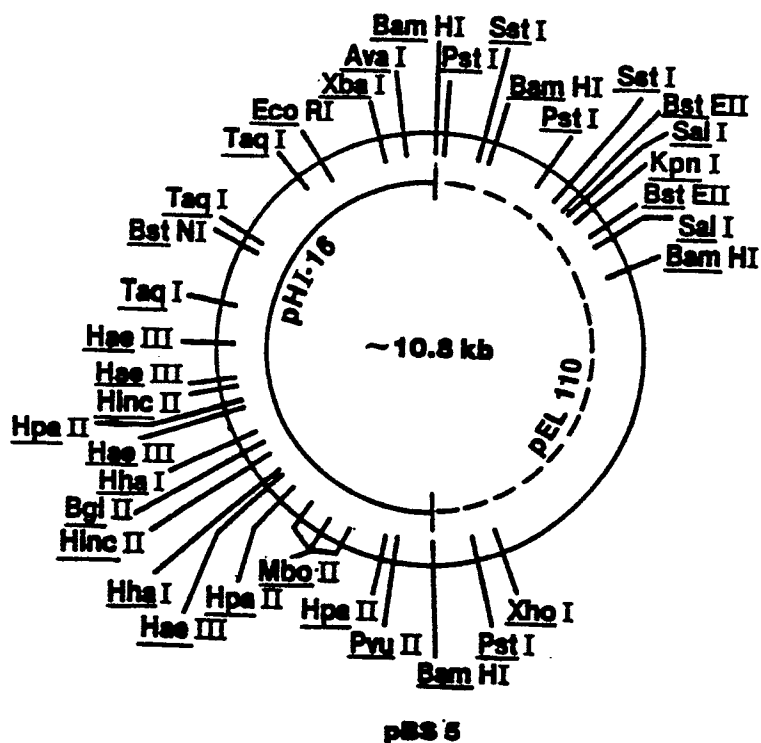


FIG. 14
Restriction Site and Functional Map
of Plasmids pBS 7 and pBS 8

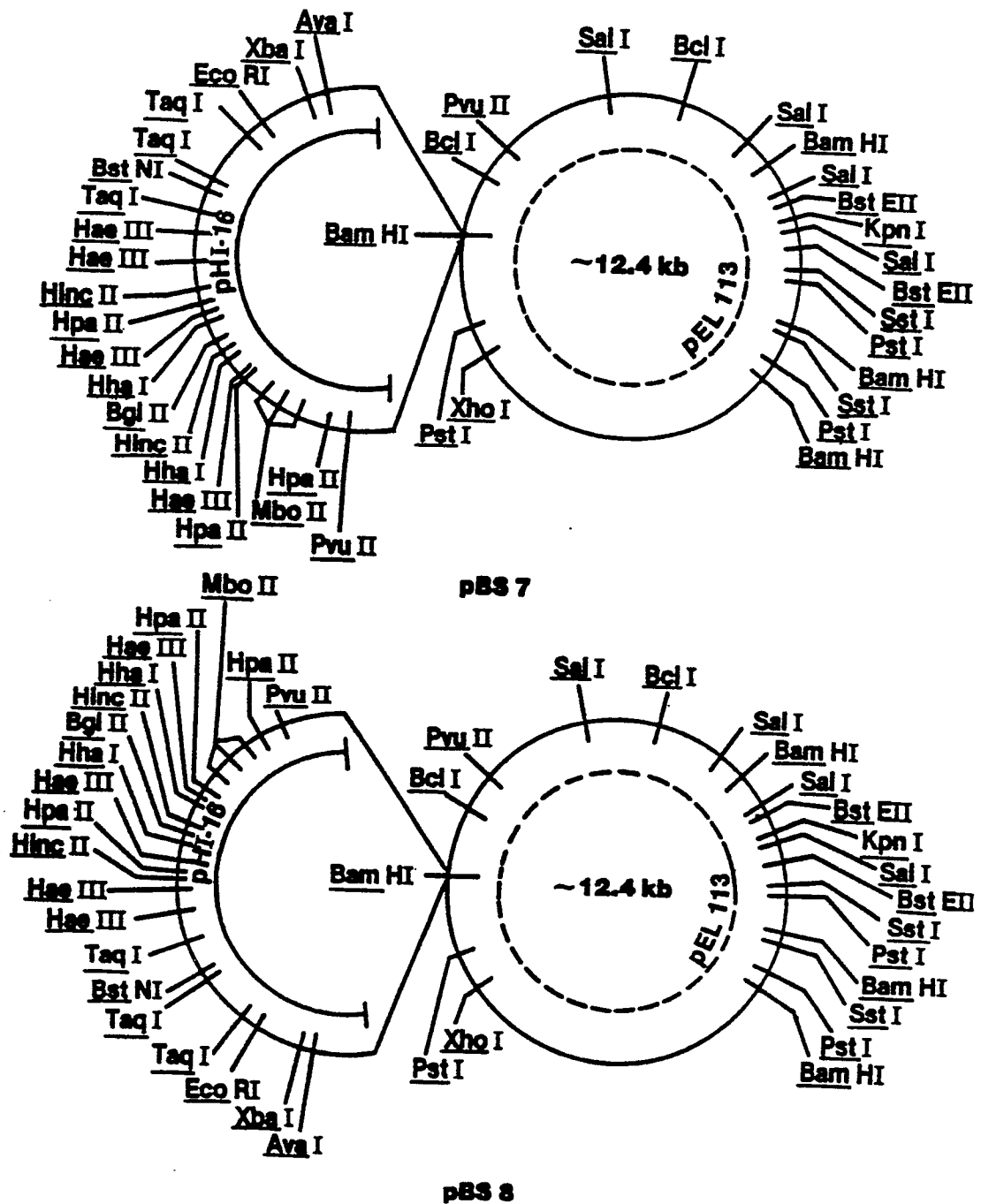


FIG.15
Restriction Site and Functional Map
of Plasmids pBS 9 and pBS 10

